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14. ABSTRACT
The primary goal of the proposed work on bone mechanical strain focuses on identifying the genes and their functions involved in mediating the anabolic skeletal response to mechanical stress. Two hypotheses have been proposed:1) Quantitative trait loci analysis using the four point bending technique in two strains of mice exhibiting extreme differences in loading response will lead to identification of chromosomal locations of genes involved in variation in skeletal response to mechanical loading. 2) Application of microarray and tyrosine phosphorylation studies using bone cells derived from inbred strains of mice exhibiting extreme differences to loading response and physiologically relevant fluid flow shear strain will lead to identification of key signaling genes and their pathways that contribute to variation in bone cell response to mechanical strain. During the last funding period, we proposed several specific objectives for each of the above-mentioned hypothesis. We have made considerable progress in accomplishing all of the specific objectives. Our work during this reporting period has resulted in one published manuscript and two abstracts. We believe that successful accomplishment of the proposed studies will provide a better understanding of the molecular mechanisms involved in identifying the genes and their function as related to mechanical stress.

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Introduction

Mechanical loading (ML) plays an important role in the maintenance of bone mass and strength. Several reports have provided evidence that mechanical loading stimulates bone formation and that immobilization or a loss of mechanical stimulation, such as bed rest or space flight, leads to a decrease in bone formation and an increase in bone loss (1, 10, 13, 14, 18, 24, 30, 31) (3, 4, 11, 17, 26, 36). Recent studies in humans have demonstrated that bone anabolic response varies widely among individuals when subjected to the same degree of mechanical load ranging from good to moderate response (7, 9, 27, 29). Analogously, experimental animals, particularly inbred strains of mice, have also shown variability with respect to mechanical loading. Studies have shown that there are greater fold changes in bone marker genes in C57BL/6J (B6) mice as compared with C3H/HeJ (C3H) mice when subjected to a same loading regimen (11). It is likely that these variations in the bone anabolic response, in both human and mouse models, are due to differences in the transcription levels of genes, i.e., they are genetically controlled.

One of the approaches often used to study the genetic regulation of an observed phenotype is QTL mapping. This approach has been well-established in both human and mouse models and has revealed hundreds of chromosomal regions containing genes affecting bone phenotypes such as BMD, bone size and bone strength (2, 15, 22, 23). Previously, using this traditional or “classical” quantitative trait loci analysis (cQTL), we have identified several loci that regulate BMD and bone size in response to mechanical loading in the B6XC3H cross (12). In order to validate these findings and to discover additional QTLs, we have used expression QTL mapping (eQTL) in the same inbred strain cross.

Recently, a number of studies in humans and animal models have provided evidence that expression levels of genes are amenable for genetic analysis in search of loci for the phenotypic variation (8, 20, 25, 28, 32, 34, 35). This eQTL approach has several advantages: 1) it can map a QTL to the gene itself, indicating whether cis changes or trans factors are responsible for the different expression levels, 2) it allows one to identify genetic regions that directly control the expression levels of genes and 3) it validates that the chromosomal region identified from cQTL analysis and determines these regions are responsible for the difference in transcription levels of genes whether responsible for the difference in bone response to loading between the two strains of mice. In the present study, we have treated expression levels of bone markers genes as quantitative traits for two inbred strains, C57BL/6J and C3H/HeJ, a good and poor responder, respectively, in order to perform a genome-wide search of loci regulating bone anabolic response to mechanical loading.

Body: The goal for the first year of this continuation proposal for the in-vivo studies for the revised Technical Objective-I, as well as our progress for each of the specific objectives in Technical Objective-I, have been addressed in previous reports.

Our specific objectives during the **first year** of this continuation proposal for the *in vivo* studies are as follows:

1) To cross two strain of mice (a poor responder strain and a good responder strain) to produce F1 mice.
2) To intercross F1 mice from these two strains to produce about 300 F2 mice.
3) To begin phenotyping the 300 F2 mice with our newly validated phenotype (i.e. realtime PCR of bone marker genes)
4) To begin genotyping the 300 F2 mice.
5)To determine the fate of new bone gained during 2 weeks of mechanical loading (i.e. to determine how long the bone density and/or bone strength gained during 2 weeks of mechanical loading is maintained after termination of 4-point bending).
6) To determine if the load applied to increase optimal anabolic response causes micro cracks in loaded bone.

This annual progress report addresses objectives 7-10.

Our goals for the last ten months of the funding period for the revised Technical Objective-I, as well as our progress for each of the specific objectives in Technical Objective-I, are described below.

Our specific objectives during the **final 10 months** of the continuation grant for the *in vivo* studies are as follows.

7) To begin phenotyping the 300 F2 mice with our newly validated phenotype (i.e. real-time PCR of bone marker genes).
8) To continue the genotyping of the F2 mice.
9) To apply MAP QTL Pseudomarker or other programs to identify genetic loci that is involved in mediating anabolic response to loading.
10) To obtain congenic strains of mice, which contain bone density and/or bone size QTL and evaluate if identified QTL region for bone density/bone size contain gene/s that contribute to bone anabolic response to mechanical loading.

Specific Objective 7: To begin phenotyping the 300 F2 mice with our newly validated phenotype (i.e. realtime PCR of bone marker genes)

After the last loading regimen, followed by in vivo pQCT measurement, mice were sacrificed, tibias were collected and store in RNA later for later RNA extraction. We used Qiagen lipid RNA extraction kit [Qiagen, Valencia, CA] to extract RNA from bones with the following modification. After euthanization, tissues were removed from test mice and stored with RNA later (a chemical that prevents degradation of RNA) at -80ºC. The autoclaved mortar and pistol were washed twice prior to our extraction, using DEPC water and cooled with liquid nitrogen (This kept the bone RNA from degrading while in the mortar).

At this point, 5-7 mm bone was added and liquid nitrogen was added three times until it froze. This caused the bone to become brittle and easier to achieve a fine powder. Approximately 1ml of Trizol was added to each sample and ground until it became a finer powder. This fine bone powder were removed from the cold mortar using a sterile razor blade and transferred quickly to a fresh 1.5 ml RNase free tube. Chloroform (200 µl) was added to each sample, and each sample was shaken (up & down) for 15 seconds and incubated at room temperature for 3 minutes (This step is highly important because long duration of incubation and forceful shaking degrade the RNA). The samples were then centrifuged at 12,000 g for 15 minutes and the aqueous layer was removed carefully to a fresh tube after centrifugation. Approximately 700 µl of ethanol was added to the fresh samples and shaken gently (up & down) for 15 seconds. The samples were then transferred to a spin column and the RNA was purified according to the manufacturer's instructions. Quality and quantity of RNA were analyzed using Bio-analyzer and Nano-drop instrumentation [Agilent]. We have completed total RNA extraction from 329 F2 mice. An excel chart has been made that contain information about the quantity, quality and mice ID for each total RNA extracted from mice [not included].

Based on our previous studies we selected two bone genes to evaluate the bone anabolic response to mechanical loading in F2 mice. These two genes, as evidenced from our study as well as from other studies, have been used to define increase or decrease in bone formation response. These genes are: 1) Bone sialoprotein and 2) alkaline phosphatase. Quantitation of messenger Ribonucleic acid (mRNA) expression was carried out according to the manufacturer's instructions (ABIPRISM, Foster City, CA.) using the SYBR Green method on 7900 Sequence Detection systems from Applied Biosystems. Briefly, purified total RNA [200µg/µl] was used to synthesize the first strand cDNA by reverse transcription according to the manufacturer's instructions [Bio-Rad, CA]. One µl of this first strand cDNA reaction was subjected to real time PCR amplification using gene specific primers. The primers were designed using Vector NTI software and were purchased from IDT-DNA. Approximately 25µl of reaction volume was used for the real time PCR assay that consisted of 1X [12.5µl] Universal SYBR green PCR master mix [Master mix consists of SYBR Green dye, reaction buffers, dNTPs mix, and Hot Start Taq polymerase] [Applied Biosystems, Foster City, CA], 50nM of primers, 24µl of water, and 1ul of template. The thermal conditions consisted of an initial denaturation at 95ºC for 10 minutes followed by 40 cycles of denaturation at 95ºC for 15 seconds (sec), annealing and extension at 60ºC for 1 minute, and a final step melting curve of 95ºC for 15 sec, 60ºC for 15 sec, and 95ºC for 15 sec. All reactions were carried out in duplicate to reduce variation. The data were analyzed using SDS software, version 2.0, and the results were exported to Microsoft Excel for further analysis.

Expression levels from each of the bone formation markers were measured as fold change by comparing the difference between loaded tibiae versus non-loaded tibiae. For gene expression studies, we used 241 samples since they represented RNA of high quality and sufficient yield. The mean fold increases in BSP and ALP, in the parents, female F1 and F2 mice, normalized by β-actin and by PPIA are shown in **Table-1**.

The fold change data for the BSP and ALP marker genes in the female F2 mice, obtained after normalizing with β-actin and PPIA, show skewed distribution (**Figure 1**). The skewed distribution appears to be due to the fold change calculation which amplifies the difference geometrically ($2^{-\Delta\Delta CT}$). Thus, by adjusting the data with natural log, the distribution became normal. The broad sense of heritability, calculated as described previously, for the loading-induced fold changes in the BSP and ALP, after normalizing with β-actin was 87% and 91% respectively, and after normalizing with PPIA was 88%, and 91%, respectively in the F2 population.

Table-1: Fold changes in the expression levels of bone marker genes in response to 12 days four-point bending on 10-week female mice.

Groups		BSP	ALP	N=
B6 Parents		8.41 ± 0.76	6.29 ± 0.71	5
C3H Parents		2.93 ± 0.62	3.38 ± 0.69	5
F1	β-actin normalized	1.87 ± 2.36	2.09 ± 2.03	16
	PPIA normalized	1.44 ± 1.82	1.82 ± 1.89	
F2	β-actin normalized	3.0 ± 2.59	2.82 ± 2.52	241
	PPIA normalized	2.83 ± 2.31	2.70 ± 2.31	

Values mentioned above are Mean ± SD of the fold change

We next determined whether the mRNA levels of bone formation marker genes (fold change), measured by real time PCR, correlate with the changes in BMD and log PC measured by pQCT. The results show a significant positive correlation between fold changes and BMD (r = 0.25 to 0.30), and bone size (r = 0.27 to 0.36). These findings suggest that these two bone formation markers are responsible, in part, for the increase in BMD and PC in response to loading in the F2 mice.

Since B6 and C3H mice exhibit difference in bone size due to their genetic background, we expect a variation in the cross-sectional area among F2 mice. Due to this variation, mice with smaller cross-sectional areas are predicted to receive a higher mechanical strain, while mice with larger cross-sectional areas to receive less mechanical strain to the same load. In order to determine if this variation in strain affected the expression levels of these markers, we performed a correlation analysis between the fold changes in BSP and ALP, normalized with β-actin and PPIA, and non-loaded PC measurements of each F2 mouse.

These results showed no correlation (r = -0.003 to 0.03). Similarly, we found no correlation between body-weight and fold change data for BSP and ALP. These data suggest that variation in the increase in mRNA levels of BSP and ALP genes induced by mechanical loading is largely independent of bone size and body weight in the B6XC3H intercross.

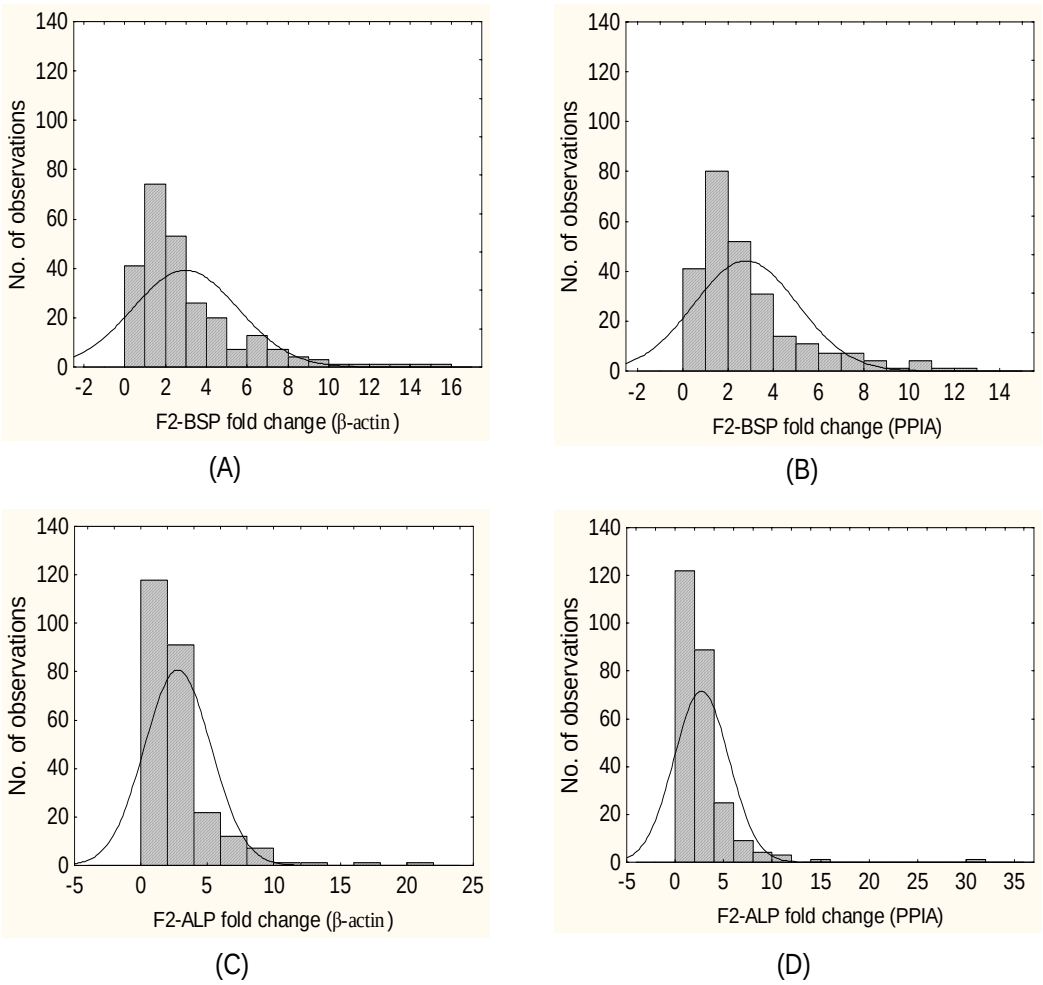

Figure 1: Distribution of fold changes for (A) BSP normalized with β-actin, (B) BSP normalized with PPIA, (C) ALP normalized with β-actin, and (D) ALP normalized with PPIA in the F2 population after two weeks of four-point bending. The x-axis represents the fold change and y-axis represents the number of observations (mice). BSP; Bone sialioprotein, ALP; Alkaline phosphatase, β-actin; Beta actin and PPIA; Peptidy-prolyl cis-trans isomerase A. The solid line represents theoretical skewed distribution. Based on kolmogorov-smirnov test, both BSP and ALP fold change data show skewed distribution (n=241).

Specific Objective 8: To continue the genotyping of the F2 mice.

We have now extracted DNA from the liver of each F2 mouse using a qiagen DNA extraction kit. The quality and quantity of the extracted DNA was measured by nano-drop and bio-analyzer. 120 Polymerase chain reaction primers was purchased from Applied Biosystems to perform the genotyping on the F2 population (n=329). All these markers were chosen depending upon the position on the chromosome in an effort to distribute then at <15cM to generate a complete genome wide scan. To proceed faster and restrict the usage of more chemicals in our genotyping reaction, we have optimized the PCR reactions (Eppendorf reagents were used) and running conditions to perform multiplexed (3-4 micro satellite markers) in a single electrophoretic lane. The pooled products were analyzed for fragments size

on the ABI 3100 Sequence Detection System and Gene Scan software was used to detect size of the alleles. Allele calls and edits were performed using Genotyper software and in house software, and exported as text files for downstream analysis.

Specific Objective 9: To apply MAP QTL pseudomarker or other programs to identify genetic loci that are involved in mediating anabolic response to loading.

We used non-parametric mapping due to skewed distribution of BSP and ALP fold change data in the F2 population. The mapping was performed by using a MapQTL software program (Verison 5.0; Wageningen, The Netherlands). The significance of the QTL were derived based on the Map QTL program as described earlier (12). Using 111 micro-satellite markers and loading induced fold change data of BSP and ALP marker genes of F2 female mice (n=241), a genome-wide analysis revealed the presence of significant and suggestive genetic loci affecting bone anabolic response (**Table-2**). Loci regulating both the expression of BSP and ALP, normalized with β-actin and PPIA, were located on Chromosomes 8, 9, 16, 17, 18 and 19. Loci regulating only BSP were located on Chrs 1, 5 and 9, whereas loci regulating only ALP were located on Chrs 1, 3 and 4. For BSP, highly significant LOD scores were observed on Chr 1[LOD score 10.4 @ 91.8], Chr 17 [LOD score 11.2 @ 14.2cM], and Chr 19 [LOD score 10.2 @ 3.3cM]. For ALP, highly significant LOD scores were observed on Chr 8 [LOD score 12.8, @ 60cM], Chr 17 [LOD score 9.3, @ 14.2] and Chr 18 [LOD score 12.6 @38cM].

Table-2: Significant and suggestive QTL identified using fold change data for the mechanical loading induced phenotypes in the B6XC3H F2 mice.

Phenotypes	Chr	Locus	cM	β-Actin Normalization	PPIA Normalization
				LOD score	LOD score
Bone sialoprotein	1	D1Mit113	91.8	-	10.4[a]
	5	D5Mit143	73.2	-	5.2[c]
	8	D8Mit88	60.1	7.5[b]	8.8[b]
	9	D9Mit2	13.1	7.0[b]	4.8[c]
	9	D9Mit151	69.9	7.1[b]	5.0[c]
	16	D16Mit153	45.9	6.7[b]	7.0[b]
	17	D17Mit51	14.2	9.2[a]	12.3[a]
	18	D18Mit144	38	8.0[b]	-
	19	D19Mit68	3.3	7.2[b]	10.7[a]
Alkaline phosphatase	1	D1Mit215	47	5.0[c]	7.5[b]
		D1Mit102	75.4	6.3[b]	7.6[b]
	3	D2Mit147	59	7.7[b]	8.3[b]
	4	D4Mit308	54.6	5.5[c]	5.6[b]
		D4Mit256	82	5.6[c]	5.0[c]
	8	D8Mit88	60.1	10.5[a]	12.3[a]
	9	D9Mit151	69.9	7.4[b]	-
	16	D16Mit153	45.9	5.1[c]	5.9[c]
	17	D17Mit51	14.2	9.3[a]	9.0[b]
	18	D18Mit144	38	12.6[a]	5.8[c]

[a]The threshold for the highly significant LOD score is $p<0.01$.
[b]The threshold for the significant LOD score is $p<0.05$.
[c]The threshold for the suggestive LOD score is $p<0.1$.
- Corresponds to no QTL.

We undertook a number of precautions to ensure that the QTLs we identified are real and not due to technical or design artifacts: 1) We chose two markers, BSP and ALP, that showed significant positive correlation in bone anabolic response to loading in a previous study (11). 2) We used fold changes rather than Ct-values to study linkages so that we would identify specific genetic changes rather than general changes. 3) We used two housekeeping genes, rather than one, to normalize the expression data in order to avoid identification of QTLs stemming from variations in RNA quality among samples.

Our linkage analysis revealed several QTLs that are responsible for the increased expression levels of BSP and ALP, induced by mechanical loading in the F2 mice. If changes in BSP and ALP markers reflect skeletal changes to mechanical loading, one would expect QTLs which are common to both markers. Accordingly, we found co-localized loci on Chrs 8, 16, 17, 18 and 19 for both BSP and ALP, suggesting that both markers are responding to the same upstream signaling.

Our findings also revealed four loci on Chrs 3, 8, 17 and 18 which are identical to QTLs we previously found for BMD and/or bone size parameters (12). This is consistent with other studies which have shown that Chrs 8 (30-90cM), 17 (6.6cM) and 18 (32-46cM) contain a loci which regulate biomechanical properties in several inbred mouse strain crosses (15, 16, 21, 22). The fact that we found QTLs at the same loci using both bone parameters and bone formation markers, indicate that these loci do, in fact, contain genes that are not only involved in increasing bone formation in response to loading, but also involved in regulating mechanical properties of the bone, in part, through ALP and BSP expression. While the expression QTLs found on Chr 17 and 18 were contained within the region identified for the BMD and bone size, the broad QTL regions in these chromosomes raise the possibility that more than one gene could be responsible for the phenotypic changes. Fine mapping will narrow down the size of the QTL and allow us to identify is as the same QTL as identified for BMD or as a different QTL. Surprisingly, we identified additional QTLs for BSP and/or ALP which do not correspond with any QTLs reported for bone parameters. This could be explained by: 1) changes in gene expression might be more sensitive to external loading than net change in the bone parameters (measured by pQCT) and 2) these regions may be involved in regulating the expression of BSP or ALP, but have no measured effect on bone formation.

To assure that the QTLs identified for BSP and ALP fold change in response to mechanical loading are not due to changes in the housekeeping genes, we calculated fold change of β-actin normalized by PPIA and PPIA fold change normalized by β-actin. We found that the mean fold difference in β-actin was 0.99 ± 0.30 and in PPIA was 1.05 ± 0.33 in the F2 mice. Interval mapping using F2 mice (n = 241) revealed four suggestive QTL on chromosomes 1, 9 and X and one significant QTL on Chr 2 (**Table-3**). We found that one of the loci on Chr 9 (69.9cM) identified for the β-actin fold change data corresponds to one of the loci identified for both BSP and ALP fold change data. This finding leads us to suspect that the QTL identified for

ALP and BSP on Chr 9 could be due to expression changes in the house keeping gene rather than due solely to expression changes in the marker genes. Thus, the validity of Chr 9 QTL remains to be established.

Table-3: Interval mapping for the fold change in β-actin (normalized by PPIA) and PPIA (normalized by β-actin) in response to mechanical loading in the B6XC3H F2 mice.

Phenotypes	Chr	Locus	cM	LOD Score	Variance
β-actin	1	D1Mit430	6.6	2.0[c]	3.6
	2	D2Mit66	48.1	2.8[b]	5.4
	9	D9Mit151	69.9	1.9	3.6
PPIA	2	D2Mit285	72.1	*	3.3
	X	DXMIt172	40.4	2.1[c]	4.7

[b]The threshold for the significant LOD score is p<0.05
[c]The threshold for the suggestive LOD score is p<0.1
* QTL with very low LOD score
Variance are explained from peak LOD Score

In addition to the mechanical loading QTL, we identified QTLs that regulate the basal expression of BSP and ALP in the non-loaded tibiae, by using Ct-values from Real time PCR, after normalization with β-actin and PPIA. Both BSP and ALP showed normal distribution in the F2 population (**Figure 2**). We found that the BSP and ALP data showed no correlation with body weight suggesting that the basal expression of these two bone formation marker genes are independent of the body weight. Interval mapping was then performed using F2 female mice (n=241), which revealed four chromosomes, Chrs 4, 10, 16, & 18 that regulate the basal expression of BSP and ALP in the non-loaded tibiae (**Table-4**).

Table-4: Significant and suggestive QTL identified using CT values for the non-externally loaded phenotypes in the B6XC3H F2 female mice.

Phenotypes	Chr	Locus	cM	LOD	Variance	LOD	Variance
				Actin Normalization		PPIA Normalization	
BSP	4	D4Mit42	76.5	2.7 [b]	5.2	2.4 [c]	4.5
	18	D18Mit64	0	2.4 [c]	4.6	*	2.3
ALP	4	D4Mit42	76.5	2.8 [b]	5.2	*	3.3
	10	D10Mit213	6.6	2.3 [c]	4.3		
	16	D16Mit153	45.9	1.9	3.6	2.3 [c]	4.2
	18	D18Mit144	38.3	2.4 [c]	6.7	*	2.3

[b]The threshold for the significant LOD score is p<0.05
[c]The threshold for the suggestive LOD score is p<0.1
* QTL with very low LOD score
Variance are explained from peak LOD Score

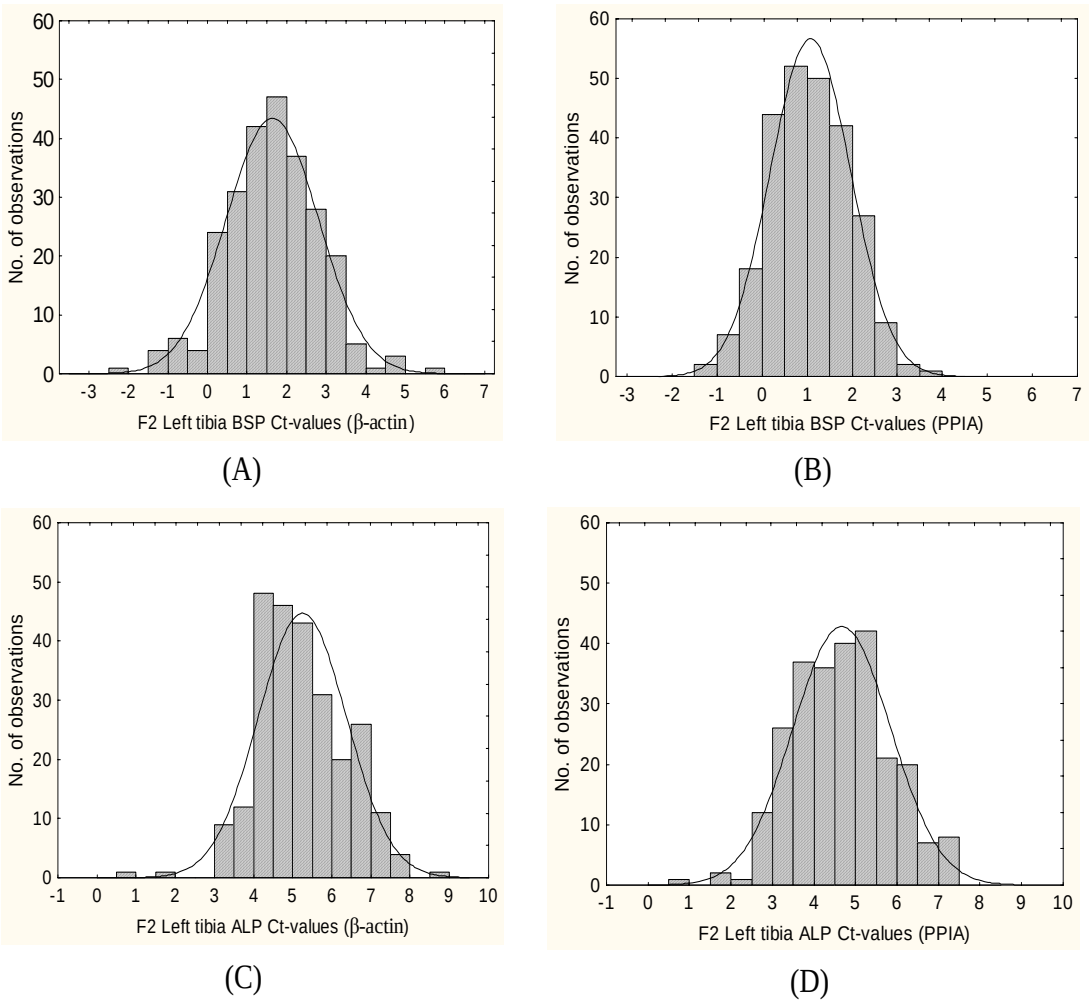

Figure 2: Distribution of Ct-values of non-external loaded tibia for (A) BSP normalized with β-actin, (B) BSP normalized with PPIA, (C) ALP normalized with β-actin and (D) ALP normalized with PPIA in the F2 population after two weeks of four-point bending. The x-axis represents the fold change and y-axis represents the number of observations (mice). BSP; Bone sialioprotein, ALP; Alkaline phosphatase, β-actin; Beta actin and PPIA; Peptidy-prolyl cis-trans isomerase A. The solid line represents theoretical normal distribution. Based on kolmogorov-smirnov test, both BSP and ALP show normal distribution (n=241).

The QTLs identified in this study common to both BSP and ALP are more than 10cM in length, and thus, contain hundreds of genes and EST's. Some of the known candidate genes located in these QTL regions is shown in **Table-5**. We have previously reported that the expression of some of these genes increase with mechanical loading using a genome-wide microarray analysis (33). Others using various approaches have shown that many of these genes are involved in skeletal development. This confirms our present QTL findings. Although QTL analysis leads to a precise mapping of the genetic loci which contribute to our phenotype of interest, these regions are broad and contain many possible significant genes. The next phase of our study, therefore, lies in identifying which specific genes within our identified QTL regions are associated with mechanical loading.

Table-5: List of potential candidate genes located in the QTL region identified for skeletal anabolic response to mechanical loading.

Chrs	cM	Genes	Predicted functions in bone
8	38-69	Ptger1	Prostaglandin E2 stimulates fibronectin through ptger1.
		Junb	Involved in osteoblast cell proliferation.
		Mt1, 2	Regulate early stage of mesenchymal stem cells differentiation.
		Cdh11	Knock out (KO) mice show reduced bone density.
		Hsd11b2	Regulate glucocorticoid signaling.
		Cdh1	Important in embryonic limb buds development.
		Cbfb	Required for the function of Runx1 and Runx2 in skeletal development.
		Hsd17b2	Involved in regulation of estrogen actin.
		Il17c	Involved in osteoclastogenesis.
16	30-46	Col8a1	Increased in bone in response to mechanical loading.
		EphA3	Involved in tooth development.
		Pit1	Mediates bone formation by regulating the expression of BSP.
17	0-25	Map3k4	Involved in normal skeletal patterning.
		Clcn7	Critical for osteoclast resorption.
		Thbs2	KO mice show increased bone density and cortical thickness.
		Traf7	Involved in MEKK3 signaling and apoptosis.
		Tnf	Important in osteoclastogenesis.
		Notch3	Involved in tooth development.
		Vegfa	Involved in angiogenesis of bone.
		Runx2	Involved in skeletal development.
18	15-40	Lox	Play a key role in the collagen deposition by osteoblast.
		Pdgfrb	Involved in bone remodeling phase.
		Adrb2	Produces anabolic effects on bone.
		Mc4r	KO mice show decreased bone resorption and high bone mass.
		Mapk4	Intracellular mediator of growth factor.
		Nfatc1	Important in osteoclastogenesis.
		Galr1	Important for bone healing.
19	0-24	Gal	Involved in bone healing.
		Lrp5	Regulates osteoblast function.
		Esrra	Important in bone metabolism.
		Lpxn	Involved in podosomal signaling complex in osteoclast.
		Ostf1	Stimulate osteoclast formation.
		Jak2	Involved in osteoblast signaling.

Some of the limitations of this study are: 1) we used a relatively small number of F2 mice to perform the eQTL analysis (n=241) relative to the cQTL analysis (n=329). This is due to fact that we encountered problems with quality and quantity in the RNA extracted from 6 mm, marrow-flushed tibiae. This relatively small sample size may account for the reduced LOD score for some of the QTLs identified in our study. 2) The QTLs identified in this study for bone formation response induced by mechanical loading were obtained from female mice. To date, studies have shown that hormones enhance the effects of mechanically induced bone

formation (5, 6, 19). Further studies with male mice may not only reveal whether any of the QTLs we found are female specific but may also lead to the identification of male specific QTLs involved in bone response to mechanical loading. 4) It has been predicted that some of the skeletal changes in the F2 mice could be due to periosteal pressure caused by four-point bending. Our previous findings that sham loading neither increased periosteal bone formation nor caused changes in expression levels of bone formation marker genes (data not shown) argue against this possibility (12).

Specific Objective 10: To obtain congenic strains of mice, which contain bone density and/or bone size QTL and evaluate if identified QTL region for bone density/bone size contain gene/s that contribute to bone anabolic response to mechanical loading.

In Previous studies we have collaborated with scientists to identify genetic loci that contribute to variation in BMD using B6-CAST and B6-C3H inbred strain crosses. In subsequent studies, we have generated B6 congenic lines of mice that carry small fragments of Chr 1 from CAST mice and Chr 4 from C3H mice that contain BMD QTL gene. In order determine if the BMD QTL gene in CAST Chr 1 and/or C3H Chr 4 could also contribute to mediating response to mechanical strain. We undertook studies to generate adequate number of congenic mice to evaluate bone anabolic response to mechanical loading. If we find evidence that one or both of these congenic lines exhibit greater bone anabolic response to mechanical loading, this would help to narrow down the location of candidate gene for mechanical loading.

Presently, we have completed four-point bending on 10-week female B6 WT and B6-CAST 1-6 congenic mice. The changes in bone parameters mesaured by in-vivo pQCT are shown in **Table-6**. The results from our study indicate that changes in bone parameters were increased significantly after 12 days of four-point bending in both female B6: CAST 1-6 congenic mice and WT B6 mice. There was no significant difference in the increase in bone size and total vBMD and/cortical vBMD between both sets of mice. Based on this finding, we conclude that CAST Chr 1 (89Mb) fragment is not critical for mechanical loading induced changes in bone parameters.

Table-6 Changes in the bone parameters measured by in-vivo pQCT after 12 days of four-point bending in 10-week female littermate mice and B6: CAST 1-6 congenic mice.

A) WT B6 mice

Bone parameters	Mean ± SD			
	Non-loaded	Loaded	p-value	% change
Total Area mm^2	2.12 ± 0.82	2.64 ± 1.04	0.01	26.0 ± 5.9
Total Mineral content	1.20 ± 0.13	1.55 ± 0.17	0.003	30.0 ± 4.25
Periosteal. Circum mm	5.13 ± 0.29	5.73 ± 0.38	0.01	12.0 ± 2.63
Total vBMD mg/ccm	696 ± 22	770 ± 22	0.0001	11 ± 4.28
Cortical vBMD mg/ccm	1072 ± 18	1110 ± 12	0.001	4.0 ± 1.96
Cortical thickness mm	0.22 ± 0.01	0.27 ± 0.01	0.0002	24 ± 5.85

N=6

B) Female B6: CAST 1-6

Bone parameters	Mean ± SD			
	Non-loaded	Loaded	p-value	% change
Total Area mm^2	2.06±0.14	2.49±0.22	0.004	24.2 ± 16
Total Mineral content mg/mm	1.15±0.06	1.51±0.14	0.0002	31.76±11.5
Periosteal. Circum mm	4.98±0.11	5.53±0.29	0.001	12.0 ±6.2
Total vBMD mg/ccm	723±35	806±37	0.006	11.27 ±2.3
Cortical vBMD mg/ccm	1076±22	1116±14	0.006	3.73±1.54
Cortical thickness mm	0.22±0.01	0.27±0.02	0.0001	25.65±5.48

N=6

Key Research Accomplishments

1. Identification of several common QTL for BMD, BSP and ALP phenotypes suggests that the skeletal response to ML is largely mediated by increased BF.
2. We show, from our both QTL study as well as from others QTL study, that Chr 8 contains genes involved in increasing bone formation induced by mechanical loading and for biomechanical properties of the bone.
3. We identified two new ML QTLs on Chrs 16 and 19 using gene expression data.
4. We show that the QTL identified on Chr 4, 16 and 18 for non-loaded BSP and ALP basal expression were identical to ML QTL suggesting that these chromosomes are responsible for both the natural variation in ALP and BSP and the increase in ALP and BSP in response to loading.
5. Using congenic approach, we show Chr 1 fragment (D1Mit216-D1Mit112, 89Mb) from CAST is not involved in regulating the bone anabolic response to loading.

Conclusion

Bone formation response varies among individuals and is strongly regulated by genetic factors, as is evident from both our previous cQTL and our present eQTL analysis. Further study, with congenic and gene KO mice may help to understand the role of specific genes at the loci we have found and could provide a basis for understanding the observed variability in bone mass accretion and maintenance, resulting from exercise, in normal healthy individuals.

Reportable Outcomes

- Kesavan C, David J Baylink, Susanna Kapoor, and Subburaman Mohan. Novel Loci Regulating Bone Anabolic Response to Loading: Expression QTL Analysis in C57BL/6JXC3H/HeJ Mice Cross, *Bone*, 41: 223-230, 2007.
- Kesavan C, D J Baylink, S Kapoor and S Mohan Identifying Mechanical Loading QTL by Gene Expression Changes for Alkaline Phosphatase and Bone Sialoprotein in C57BL/6J (B6) X C3H/HeJ (C3H) Intercross. ASBMR 29th Annual Meeting 2007 Hawaii, USA.

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Molecular Genetic Studies on Bone Mechanical Strain ~ *In-vitro studies*

Introduction

This portion of the report summarizes our progress made during the past year on the identification of leptin receptor as a potential negative mechanosensitivity gene and the potential molecular mechanism whereby leptin receptor acts to suppress the fluid shear stress-induced osteoblast proliferation and differentiation.

Technical Objectives: The original specific objectives for the in vitro studies during the past year of this grant are as follows:

1. To optimize the in vitro siRNA techniques for the application to mouse osteoblasts in conjunction with our shear stress technology.
2. To select one or more ESTs (or known genes) for further study from our microarray data.
3. To apply the siRNA technique to suppress the candidate EST expression or known gene and then evaluate the functional role of this EST or known gene in osteoblast proliferation, differentiation, and apoptosis.
4. To continue to advance our protein-tyrosine phosphorylation studies in order to identify signaling proteins that show differences in protein-tyrosine phosphorylation levels in response to mechanical strain in bone cells isolated from those mouse strains which exhibit differential responses to the in vitro mouse strains. Changes in protein-tyrosine phosphorylation levels will be compared to in vitro parameters of osteoblast proliferation, differentiation, and apoptosis.

Body: In our original experimental approach, we plan to identify potential candidate genes from our microarray data and to determine the effect of suppression of expression of the candidate gene in osteoblasts by the siRNA technology on their anabolic response to fluid shear stress. From our microarray and subsequent studies, which have been included in the previous report, we have compelling evidence that four anabolic signaling pathways, i.e., the IGF-I, estrogen receptor, Wnt, and BMP/TGFβ signaling pathways are downstream to the "mechanosensitivity" genes contributing to the good and poor bone formation response in B6 and C3H mice, respectively (1), and that the leptin receptor gene may be one of the "mechanosensitivity" modulating gene located with the chromosome 4 QTL region that contributes in part to the differential anabolic response to mechanical loading in C57BL/6J (B6) and C3H/HeJ (C3H) mice. During the past year, we continued to work on testing the hypothesis that leptin receptor is a negative mechanosensitivity modulating gene and to determine potential molecular mechanisms whereby leptin receptor acts to modulate mechanosensitivty using the fluid shear stress model. The modified Technical Objectives for the past year are: 1) to further determine if leptin receptor is a negative mechanosensitivity modulating gene in primary mouse osteoblasts, and 2) to determine the molecular mechanism (i.e., mechanotransduction) whereby the candidate gene acts to regulate the anabolic response in osteoblasts.

Specific Objective 1. To further determine if leptin receptor is a negative mechanosensitivity modulating gene in osteoblasts. In the previous report, we have provided strong evidence that leptin receptor or its signaling pathway is a negative mechanosensitivity modulating gene or pathway. Accordingly, we have shown that adult female leptin-deficient *ob*/*ob* mice showed an enhanced bone formation response in tibia after a 2-week four-point bending exercise regimen compared to age- and sex-matched wild-type B6 mice at an equivalent loading strain. We also showed that the 30-min steady fluid shear of 20 dynes/cm^2 produced significantly greater increases in [^{3}H]thymidine incorporation and Erk1/2 phosphorylation in ob$^-$/ob$^-$ osteoblasts than those in B6 osteoblasts and osteoblasts of WT ob$^+$/ob$^+$ littermates, supporting the interpretation that deficiency in leptin signaling led to an enhance anabolic response to fluid shear stress (a surrogate of mechanical loading) in primary mouse osteoblasts. Moreover, we also showed that the enhanced mitogenic response to fluid shear in *ob*/*ob* osteoblasts was completely obliterated by the leptin treatment. In addition, we showed that the shear stress-mediated upregulation of gene expression of the four aforementioned anabolic pathways was significantly greater in ob/ob osteoblasts than those in B6 osteoblasts and that the 2-hr leptin pretreatment completely abolished the enhanced response in the shear stress-induced upregulation of expression of these genes. These preliminary data together suggest that the *Lepr* signaling has a negative regulatory role in the context of mechanotransduction. These *in vitro* data are also consistent with the *in vivo* loading data that deficiency of leptin expression or leptin receptor signaling enhanced the osteogenic response to mechanical stimulation.

While these preliminary findings are exciting and support our overall hypothesis, it is also somewhat puzzling to observe an enhanced mitogenic response in the isolated *ob*/*ob* osteoblasts to the fluid shear, since the *ob*/*ob* osteoblasts were deficient only in leptin production but not in the leptin receptor expression. A potential explanation for this observation is that primary B6

mouse osteoblasts, especially after exposure of the fluid shear, may produce sufficient amounts of leptin to suppress the leptin receptor signaling. The lack of the leptin production in *ob*/*ob* osteoblasts may thus alleviate the suppressive effects of the endogenous leptin, resulting in an enhanced mitogenic response to mechanical stimulation. Leptin is primarily synthesized by adipose tissues. Therefore, we next tested whether primary mouse osteoblasts may produce significant amounts of leptin, especially after the fluid shear and whether the differential osteogenic response between C3H and B6 osteoblasts is due to a different leptin secretion level by measuring the amounts of leptin protein in the conditioned medium (CM) of B6 and C3H osteoblasts 24 hrs after the 30-min fluid shear were measured with a mouse leptin EIA kit (**Fig. 1**). The concentration of leptin in CM of each static control culture was detectable albeit low (~2 pg/mg cellular protein/24 hr), confirming that leptin is secreted by primary osteoblasts (2). The fluid shear increased the CM leptin concentration by ~3-fold in either osteoblast culture, but there was no significant difference. Thus, we conclude that the differential osteogenic response to shear stress between B6 and C3H osteoblasts was not due to a different leptin secretion or production level between the two osteoblasts. This raises the interesting possibility that the effects were due to significant differences in leptin receptor and/or its signaling pathways between B6 and C3H osteoblasts.

In this regard, we reported in the previous progress report that there was no significant difference in the basal or the shear stress-induced leptin receptor mRNA expression levels in C3H and B6 osteoblasts, indicating that the reported differences were not due to differences in leptin receptor expression levels. However, we have identified three single nucleotide polymorphisms (SNPs) in the open reading frame of the leptin receptor gene between B6 and C3H osteoblasts. Two of the SNPs are silent and are located at the wobble base, but the A→G SNP at nucleotide 1075 yielded an Ile→Val substitution at amino acid residue 359. Although the physiological significance of this substitution is unclear, we postulate that this substitution may affect the ligand binding and,

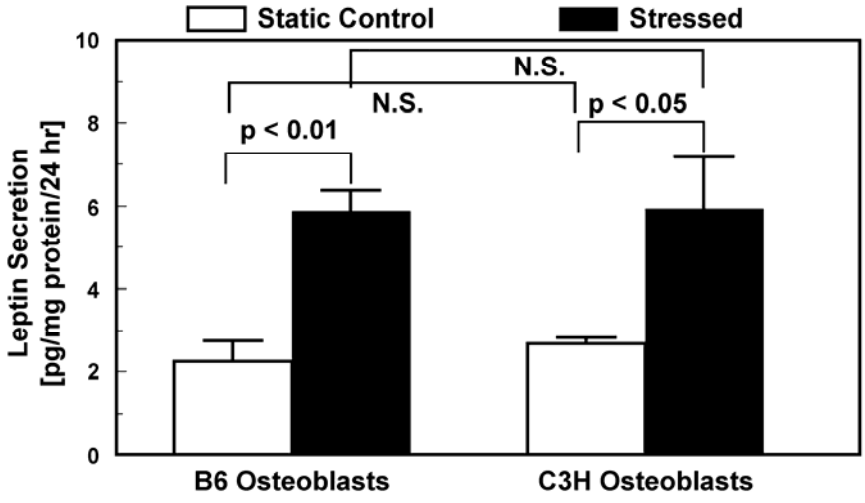

Figure 1. Comparison of basal and shear stress-induced secretion of leptin by B6 and C3H osteoblasts in vitro. The 24-hr conditioned media (CM) of B6 (left) or C3H osteoblasts (right) with or without the 30-min fluid shear were concentrated 7-fold. The amounts of leptin in each concentrated CM was assayed with the mouse leptin EIA kit (Assay Designs, Inc.) and normalized against cellular protein content. Results are shown as mean ± SD (n = 4 each).

thus, the leptin receptor signaling, since the substitution is located within the ligand binding domain.

To further determine if leptin receptor or its signaling is a negative regulatory mechanism of mechanotransduction, the effects of siRNA-mediated knockdown of leptin receptor expression on the anabolic actions of the fluid shear in B6 osteoblasts were assessed. A set of 3 *Lepr* siRNA was obtained from Qiagen: *Lepr* siRNA1 (target sequence: CCC GAG CAA ATT AGA AAC AAA), *Lepr* siRNA2 (target sequence: ATC GAT GTC AAT ATC AAT ATA), and *Lepr* siRNA3 (target sequence: TTG AAG CTA AAT TTA ATT CAA). The 24-hr treatment with *Lepr* siRNA2 reduced cellular leptin receptor protein level in B6 osteoblasts by >70% (**Fig. 2A**), and increased basal (static control) and fluid shear-induced Erk1/2 phosphorylation (**Fig. 2B**).
These data support our contention that leptin receptor or its signaling is a negative regulatory mechanism in the context of mechanotransduction.

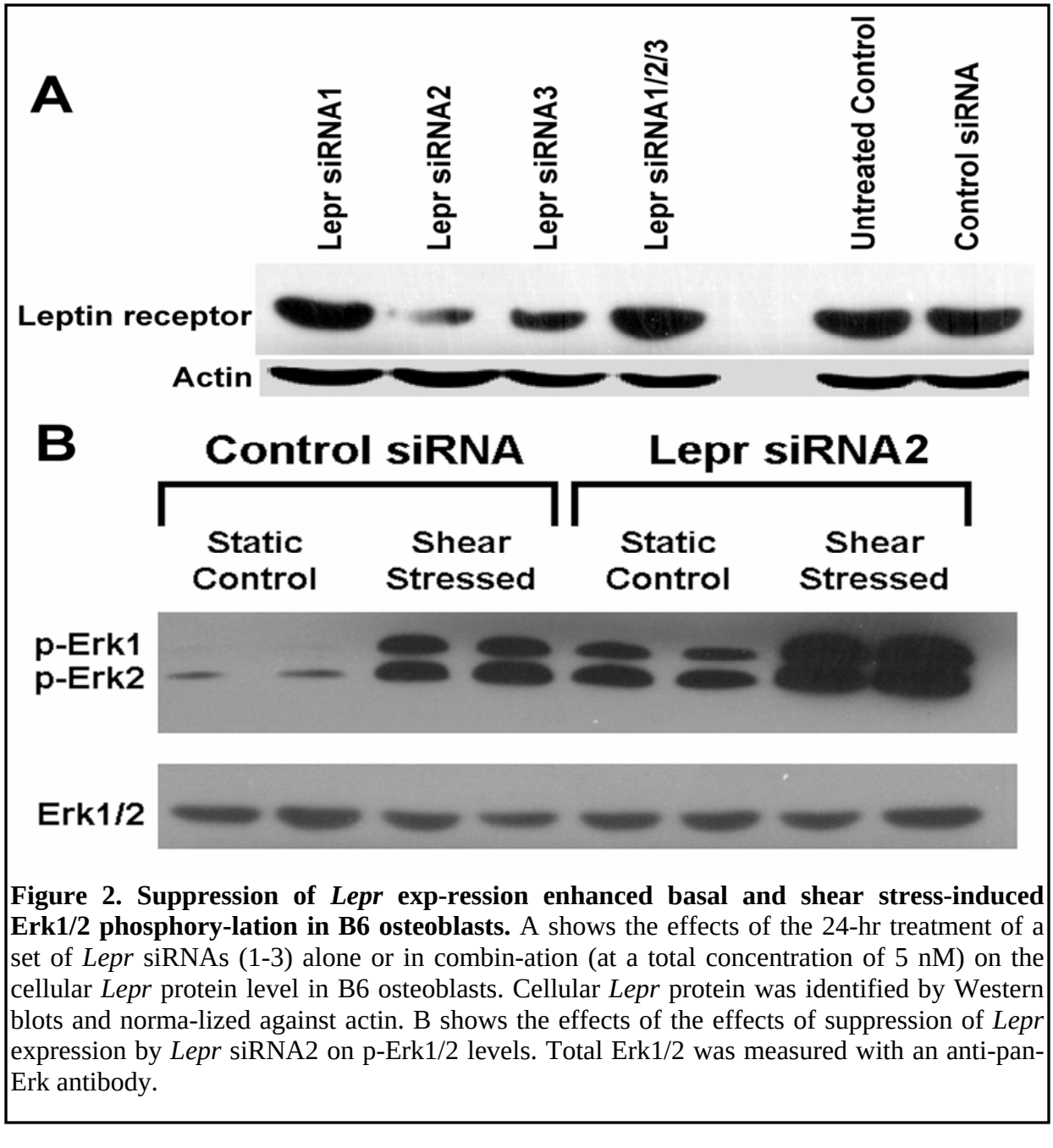

Figure 2. Suppression of *Lepr* exp-ression enhanced basal and shear stress-induced Erk1/2 phosphory-lation in B6 osteoblasts. A shows the effects of the 24-hr treatment of a set of *Lepr* siRNAs (1-3) alone or in combin-ation (at a total concentration of 5 nM) on the cellular *Lepr* protein level in B6 osteoblasts. Cellular *Lepr* protein was identified by Western blots and norma-lized against actin. B shows the effects of the effects of suppression of *Lepr* expression by *Lepr* siRNA2 on p-Erk1/2 levels. Total Erk1/2 was measured with an anti-pan-Erk antibody.

If the lack of an osteogenic response in C3H osteoblasts is due to a "defective" leptin receptor signaling, it follows that suppression of expression leptin receptor in C3H osteoblasts restores their sensitivity to mechanical stimulation of cell proliferation. Indeed, **Fig. 3** reveals that the siRNA-mediated knockdown leptin receptor expression in C3H osteoblasts restored their ability to respond to the fluid shear with an increase in cell proliferation, suggesting that a "hyperactive" leptin receptor or its signaling in C3H osteoblasts plays a contributing role in the poor mechanosensitivity in this mouse strain. A major function of the leptin receptor signaling

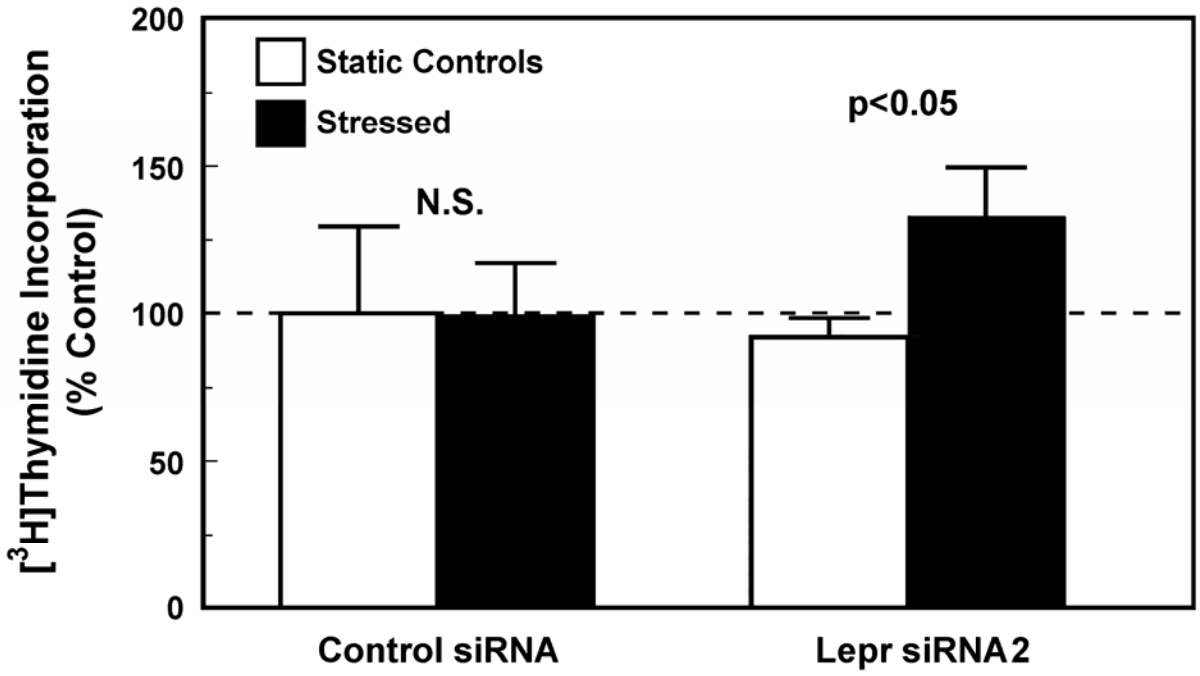

Figure 3. Suppression of *Lepr* expression restored the mechano-sensitivity of C3H osteoblasts to fluid shear-induced cell proliferation. Suppression of *Lepr* expression in C3H osteoblasts was accomplished by a 24-hr treatment with 5 nM *Lepr* siRNA2. Cell proliferation was measured by [^{3}H]thymidine incorporation during the final 6 hrs of a 24-hr incubation. Results are shown as mean±SEM (n=4 each).

is to control body fat and metabolism. Thus, if the leptin receptor signaling in C3H mice is indeed more active than that in B6 mice, there should be a significant difference between these two mouse strains in their ability to regulate body fat. Consistent with the premise of a "hyperactive" leptin receptor signaling in C3H mice, recent studies show that C3H mice have decreased adiposity and less susceptibility for body fat gain upon a high fat diet than other inbred mouse strains, including B6 mice (3-5). Consequently, these siRNA studies strongly suggest that the leptin receptor signaling in C3H osteoblasts plays an important role in their poor response to mechanical stimulation.

Specific Objective 2: Determination of the molecular mechanism whereby leptin receptor acts to negatively modulate the mechanical stimulation of osteoblast proliferation and differentiation. The mechanism(s) whereby the leptin receptor signaling pathway negatively

regulates mechanotransduction in osteoblasts is unknown. In the previous progress report, we have postulated that the negative modulating action of the leptin receptor signaling on mechanotransduction is mediated a competition between integrin β1 and leptin receptor for recruitment of SHP2 and/or PTP1B (indicated by the thick arrows in **Fig. 4**), and that the leptin receptor signaling pathway inhibits the mechanical stimulation of osteoblast proliferation in part through sequestering SHP2 and/or PTP-1B from binding to integrins. We further postulate that the mechanism(s) leading to the preferential recruitment of SHP2 and PTP-1B to integrins in response to mechanical stimuli is defective in C3H osteoblasts. In this regard, leptin receptor is a member of the class I cytokine receptor family (9) and uses Janus kinase (JAK)2-signal transducers and activators of transcription (STAT) 3 as the primary signaling (**Fig. 4**). The binding of leptin to leptin receptor results in transphosphorylation and activation of JAK2 and the subsequent phosphorylation of tyrosine residues in the cytoplasmic part of leptin receptor, which provide docking sites for SH2-containing signaling proteins, including STAT3, SHP2, suppressor of cytokine signaling 3 (SOCS3), and PTP1B. Recruitment of STAT3 to the

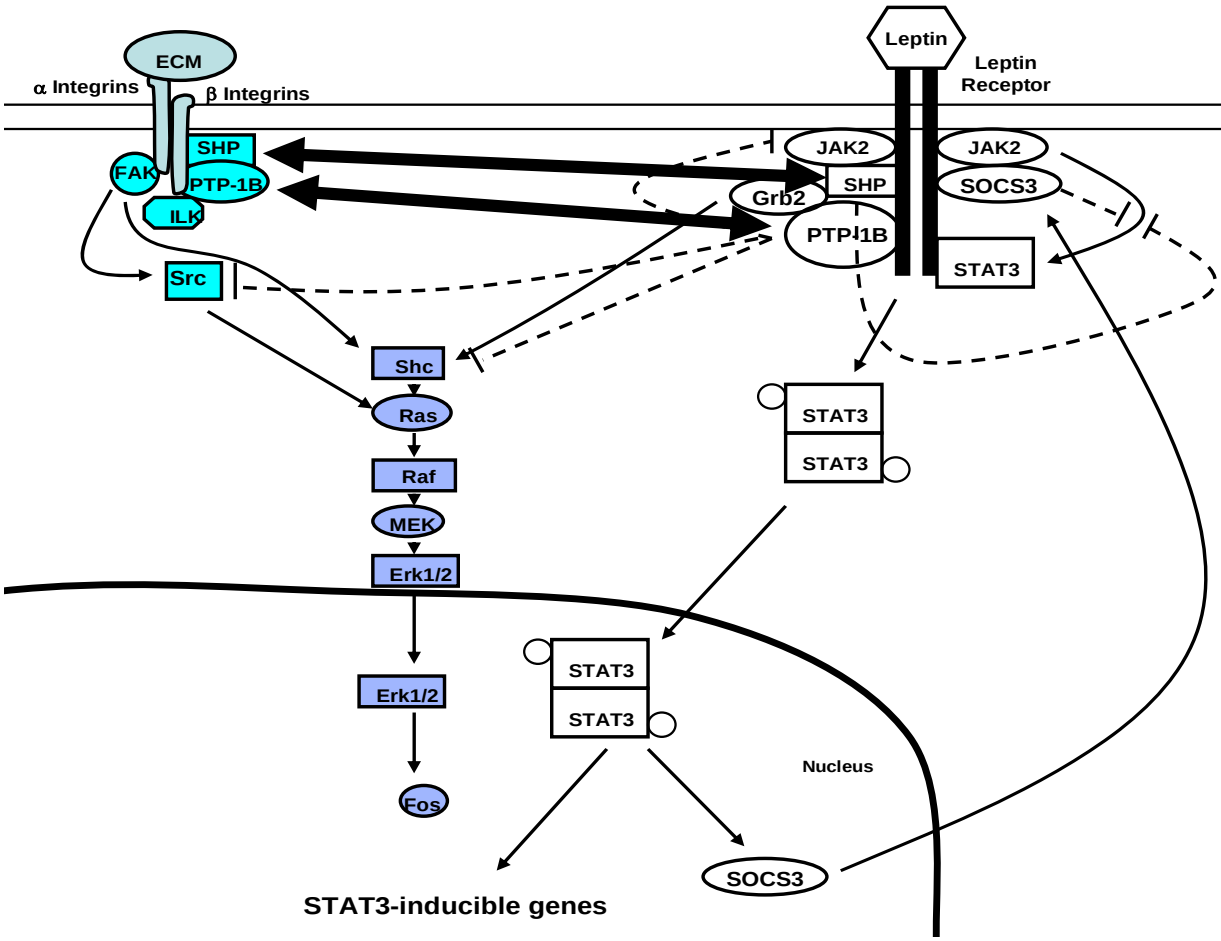

Figure 4. Molecular mechanism of the leptin receptor signaling pathway and its potential interactions with the integrin signaling pathway. Please refer to text for a detailed description. The solid lines represent upregulation; while the dashed lines indicate negative regulation. The thick arrows indicates shuffling of SHP2 and/or PTP1B between Lepr and integrins.

phosphorylated tyr(pY)-1138 residue leads to its rapid phosphorylation, dimerization, and translocation to the nucleus to activate transcription of STAT3-inducible genes, including SOCS3. The pY-985 residue recruits either SHP2 or SOCS3. Binding of SOCS3 attenuates leptin signaling by inhibiting the receptor-associated JAKs (6). Recruitment of SHP2 can have both positive and negative effects on the leptin receptor signaling. On the one hand, the SHP2 binding results in binding of Grb2 to SHP2 and subsequent activation of the Ras/Raf/Erk signaling pathway, leading to, among others, the expression of c-fos. On the other hand, SHP2 dephosphorylates and inactivates JAK2, leading to termination of the leptin receptor signaling. PTP1B is a critical downstream negative regulator of the leptin receptor pathway. Deletion of PTP1B gene enhanced leptin sensitivity in mice (7). PTP1B decreased JAK2 phosphorylation and blocked leptin-induced transcription of SOCS3 and c-fos (8). The leptin receptor signaling pathway also regulates other key anabolic pathways through crosstalks, including the integrin, IGF-I, ER, BMP/TGFβ, and canonical Wnt signaling pathways. **Fig. 4** shows some of the interactions between the *Lepr* and the integrin signaling pathways. Specifically, the recruitment of SHP2 and/or PTP1B to integrin is essential for the integrin signaling (9,10). Recruitment of these PTPs would lead to c-Src activation (by dephosphorylating its inhibitory pY-527 residue), which in turn activates the Grb2/Ras/RAF/Erk signaling. We surmise that there is a competition between integrins and leptin receptor for recruitment of SHP2 and/or PTP1B (indicated by the thick arrows in **Fig. 4***)*, and that the leptin receptor pathway inhibits the mechanical stimulation of bone formation in part through sequestering SHP2 and/or PTP-1B from binding to integrin (particularly integrin β1, the most abundant integrin in osteoblasts). We further postulate that the mechanism(s) leading to the preferential recruitment of SHP2 and PTP-1B to integrin β1 in response to mechanical stimuli is defective in C3H osteoblasts.

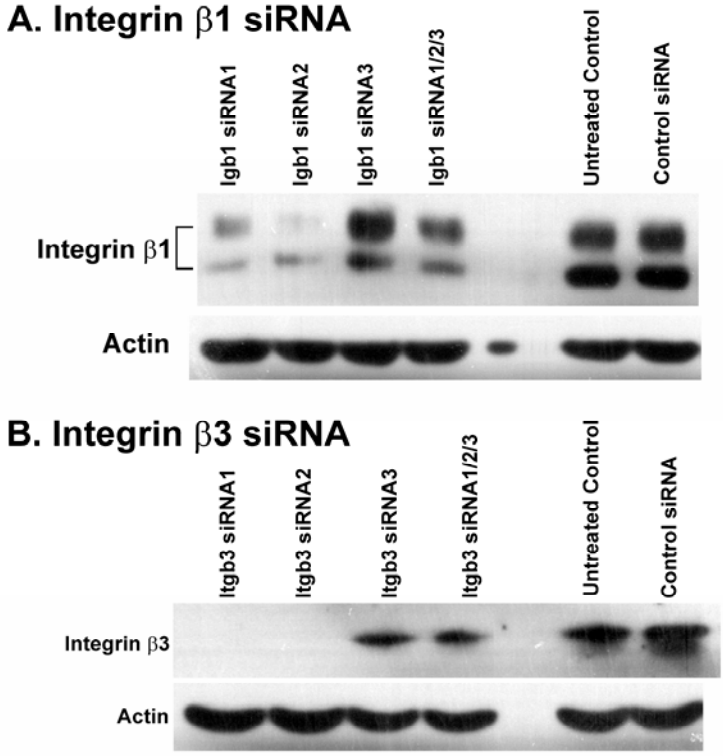

Figure 5. Suppression of integrin β1 (1) and β3 (B) expression by specific siRNAs in primary B6 osteoblasts. A set of 3 siRNAs each against mouse integrin β1 or β3 along with a control siRNA were designed and synthesized by Qiagen. B6 osteoblasts were trans-fected with each siRNA alone or in com-bination using the HiPerFect transfection reagent (Qiagen) with the total siRNA concen-tration in each treatment was 5 nM. After 24 hr, cellular integrin β1 or β3 and actin levels were analyzed by Western blots.

As a test of our model, we first sought to demonstrate that integrin

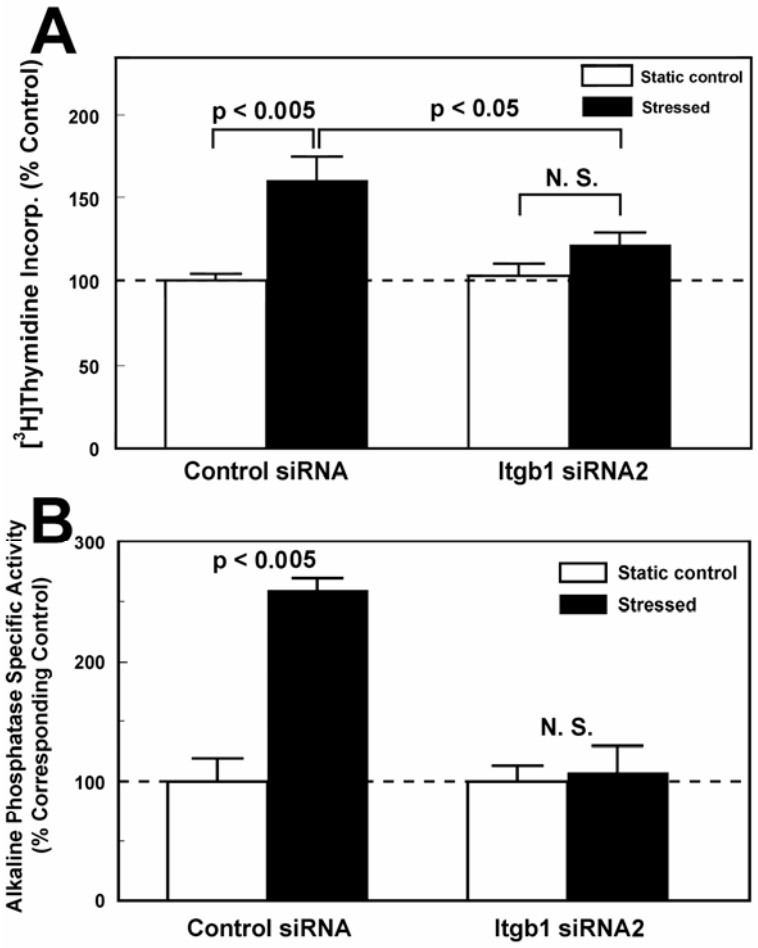

Figure 6. siRNA-mediated integrin β1 gene knockdown in B6 osteoblasts blocked fluid shear-induced prolifera-tion (A) and differentiation (B). Sup-pression of integrin β1 expression in B6 osteoblasts were done with 5 nM Itgb1 siRNA2 for 24 hrs. Results are shown as mean±SEM (n=7 for thymidine incorporation, and n=4 for ALP specific activity).

β1 signaling is indeed essential for the mechanical stimulation of osteoblasts. We suppressed integrin β1 expression in B6 osteoblasts by a specific siRNA and assessed the effects on the fluid shear-induced proliferation and differentiation. The siRNAs were pre-designed by Qiagen based on the proprietary HiPerformance siRNA Design Algorithm. A set of three siRNAs were obtained: *Igtb1* siRNA1 (target sequence: CTG CTA ATA AAT GTC CAA ATA), *Igtb1* siRNA2 (target sequence: CTG GTC CAT GTC TAG CGT CAA), and *Igtb1* siRNA3 (target sequence: CCA GCT AAT CAT CGA TGC CTA). Two of the test siRNAs (i.e., *Igtb1* siRNA1 and 2) were effective in suppressing integrin β1 expression. The 24-hr pretreatment with *Itgb*1 siRNA2 suppressed integrin β1 expression by >90% (**Fig. 5A**) and blocked the fluid shear-induced proliferation, and differentiation (**Fig. 6**). <u>These data afford indisputable evidence that integrin β1 is essential in mechanotransduction, at least in mouse osteo-blasts</u>. We also successfully knocked down integrin β3 expression [with *Itgb3* siRNA1 (target sequence: CCG CTT CAA TGA AGA AGT GAA) and *Itgb3* siRNA 2 (target sequence: CAG AGG ATT GTC CTT CGA CTA), but not *Itgb3* siRNA3 (target sequence: CGC CGT GAA TTG TAC CTA CAA)] in B6 osteoblasts (**Fig. 5B**). We will test whether integrin β3 also plays a critical role in mechanotransduction by the siRNA approach in the future.

One of our previous interesting observations concerning the mechanotransduction mechanism in B6 and C3H osteoblasts is that the basal expression of integrin β1 and Cox-2 in the mechano-non-responsive C3H osteoblasts was significantly elevated (1), questioning the

presumed roles of these mechanoresponsive genes in mechanotransduction. In this regard, our studies strongly indicate that the leptin receptor signaling is a negative mechanosensitivity modulating mechanism and we also showed preliminary evidence in the previous progress report that the leptin receptor signaling in C3H osteoblasts appeared to be significantly greater than that in B6 osteoblasts. Because one of the SNP in the leptin receptor gene between C3H and B6 osteoblasts is an nonsynonymous SNP that leads to an I359V substitution at nucleotide 1075, we postulate that the I359V substitution might yield a "hyperactive" leptin receptor. Accordingly, we hypothesize that the higher basal expression of these mechano-responsive genes (e.g., integrin β1 and Cox-2) could be due to a "feedback" up-regulation due to a "malfunctioned" (i.e., hyperactive) leptin receptor signaling.

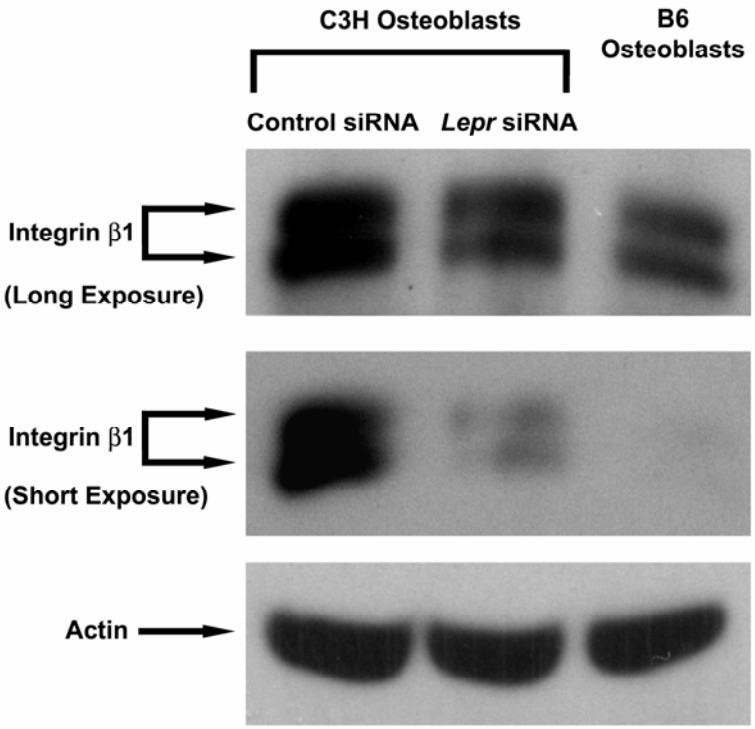

Figure 7. siRNA-mediated knockdown of *Lepr* gene expression in C3H osteoblasts markedly reduced basal integrin β1 protein expression. C3H osteoblasts treated with either the control siRNA or *Lepr* siRNA. Twenty-four hrs after the treatment, equal aliquots of cellular proteins of the treated C3H osteoblasts and the untreated B6 osteoblasts were separated on 10% SDS-PAGE and transblotted to nitrocellular membrane. The relative levels of integrin β1 were analyzed by Western immunoblot using an anti-integrin β1 antibody. The top panel shows the Western blot with 3-min exposure, and the middle panel shows the same blot with 1-min exposure. The blot was then striped and reblotted against actin (the bottom panel).

If this hypothesis is correct, we should expect that suppression of leptin receptor expression in C3H osteoblasts should lead to marked reduction of basal expression of integrin β1. To test this prediction, we have preformed a gene-specific siRNA-mediated gene knockdown experiment to assess the consequence of suppression of leptin receptor in C3H osteoblasts on basal integrin β1 protein expression levels. Briefly, C3H osteoblasts were treated with *Lepr* siRNA2 (see above) or the control siRNA (provided by the supplier) for 24 hrs as described in the parental grant, and integrin β1 protein level was analyzed by Western blot using an anti-integrin β1 antibody and normalized against actin level. **Fig. 7** confirms that the basal integrin β1 levels in C3H osteoblasts (i.e., the control siRNA-treated C3H osteoblasts) were several-fold higher than those in B6 osteoblasts. The 24-hr *Lepr* siRNA treatment drastically reduced the basal integrin

β1 protein expression level to a level approaching to that of B6 osteoblasts. This reduction is more obvious when the blot was under-exposed (middle panel of **Fig. 7**). These results clearly indicate that the higher basal integrin β1 protein expression level in C3H osteoblasts is related to *Lepr* or its signaling, and as such, are consistent with our hypothesis that the higher basal integrin β1 expression in C3H osteoblasts may be due to a "feedback" upregulation of integrin β1 expression in response to the presumably "hyperactive" leptin receptor signaling. We are currently evaluating whether the gene-specific siRNA-mediated knockdown of leptin receptor expression in C3H osteoblasts would also reduce the observed elevated basal expression levels of other key mechano-signaling genes, such as Cox-2 (1). If our hypothesis is correct, knocking down leptin receptor expression in C3H osteoblasts should also reduce the basal expression levels of Cox-2 in a similar manner.

If the I359V substitution indeed alters the efficiency of the leptin receptor signaling in C3H osteoblasts, the basal and/or shear stress-induced, leptin-mediated activation of the leptin receptor signaling (i.e., JAK2/STAT3 activation) in the two osteoblasts would be very different. Consistent with this speculation, we reported in our previous progress report that the basal and the shear stress-mediated, leptin-dependent increases in pY-JAK2 and pY-STAT3 levels in the C3H osteoblasts were each higher than those in the B6 and ob^-/ob^- osteoblasts. Because there was not a big difference in the leptin receptor mRNA expression level (shown in the previous report) and/or a difference in the production of leptin protein (**Fig. 1**) between the two mouse osteoblasts, we tentatively conclude that that the I359V substitution in the leptin receptor gene of C3H osteoblasts leads to an enhanced basal and shear stress-mediated, leptin-dependent activation of the leptin receptor signaling.

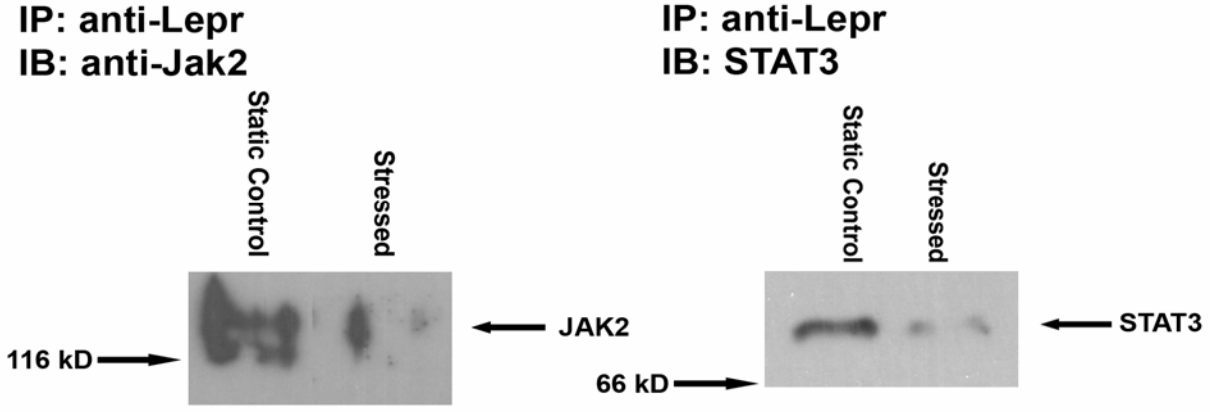

Figure 8. Effects of shear stress on the amounts of co-immunoprecipitated JAK2 and STAT3 with *Lepr* in B6 osteoblasts. Immediately after the 30-min shear stress, cell extracts of the stressed and static control B6 osteo-blasts were immunoprecipitated (IP) with anti-*Lepr* and blotted (IB) against anti-Jak2 (left panel) or anti-STAT3 (right panel).

As reported in the previous progress report, the total JAK2 and STAT3 levels were several folds higher in C3H osteoblasts than in B6 and *ob*$^{-}$/*ob*$^{-}$ osteoblasts. Inasmuch as the fluid shear suppressed the leptin-mediated pY-JAK2 and pY-STAT3 levels in C3H osteoblasts, the actual pY-JAK2 and pY-STAT3 levels were still several folds higher than the basal pY-JAK2 and pY-STAT3 levels in B6 and *ob*$^{-}$/*ob*$^{-}$ osteoblasts. The elevated basal levels of total JAK2/STAT3 in C3H osteoblasts may merely represent a physiological feedback upregulation in response to a "hyperactive" leptin receptor signaling, and the shear stress produced by physiologically relevant levels of loading might be insufficient to suppress the leptin receptor signaling to a level that would allow an anabolic response. Past findings that high levels of mechanical strains were able to elicit an osteogenic response in C3H mice (11) are in congruent with this possibility. On the other hand, we cannot rule out the possibilities that 1) the greater basal total JAK2/STAT3 protein levels (rather than the greater functional activity of leptin receptor) in C3H osteoblasts is responsible for the overall increased in the pY-JAK2/pY-STAT3 levels compared to B6 osteoblasts, and 2) other genetic differences between B6 and C3H mice are responsible for the greater basal levels of total JAK2 and STAT3 in C3H osteoblasts, which is in part responsible for the differential osteogenic response to mechanical stimulation in the two inbred mouse strains. These possibilities will be addressed in the future.

Other members of the class I cytokine receptor family may also activate the JAK2/STAT3 signaling. To ensure that the fluid shear-mediated reduction in the JAK2/STAT3 activation is related to the shear stress-mediated reduction of the leptin receptor signaling, we performed a preliminary co-immunoprecipitation experiment to determine the amounts of leptin receptor-associated JAK2/STAT3 in B6 osteoblasts (**Fig. 8**). Briefly, after the shear shear, an aliquot (0.5 μg protein) of cell extract protein of the stressed and static control B6 osteoblasts was each immunoprecipitated with an anti-leptin receptor antibody. The co-immunoprecipitated JAK2 was identified on Western blot using an anti-JAK antibody (left panel). The stripped blot was rebloted against an anti-STAT3 antibody (right panel). The fluid shear markedly reduced the amounts of leptin receptor-bound JAK2 and STAT3; a finding consistent with our interpretation that the reduced JAK2/STAT3 activation is related to the shear stress-mediated reduction in the leptin receptor signaling.

As an initial test to assess our hypothesis that the leptin receptor pathway inhibits the mechanical stimulation of bone formation in part through sequestering SHP1/2 and/or PTP-1B from binding to integrin β1, we performed a preliminary co-immunoprecipitation experiment to assess the feasibility of this approach and also to determine if fluid shear increases the amounts

of the integrin β1-associated SHP1 in B6 and C3H osteoblasts. Accordingly, extract proteins of replicate slides of static control or shear stressed osteoblasts were co-immunoprecipitated with an anti-mouse integrin β1 antibody. The presence of integrin β1-associated SHP1 was then identified by Western immunobloting using an anti-SHP1 antibody. **Fig. 9** shows that appreciable amounts of SHP1 were found to be co-immunoprecipitated with integrin β1 in both

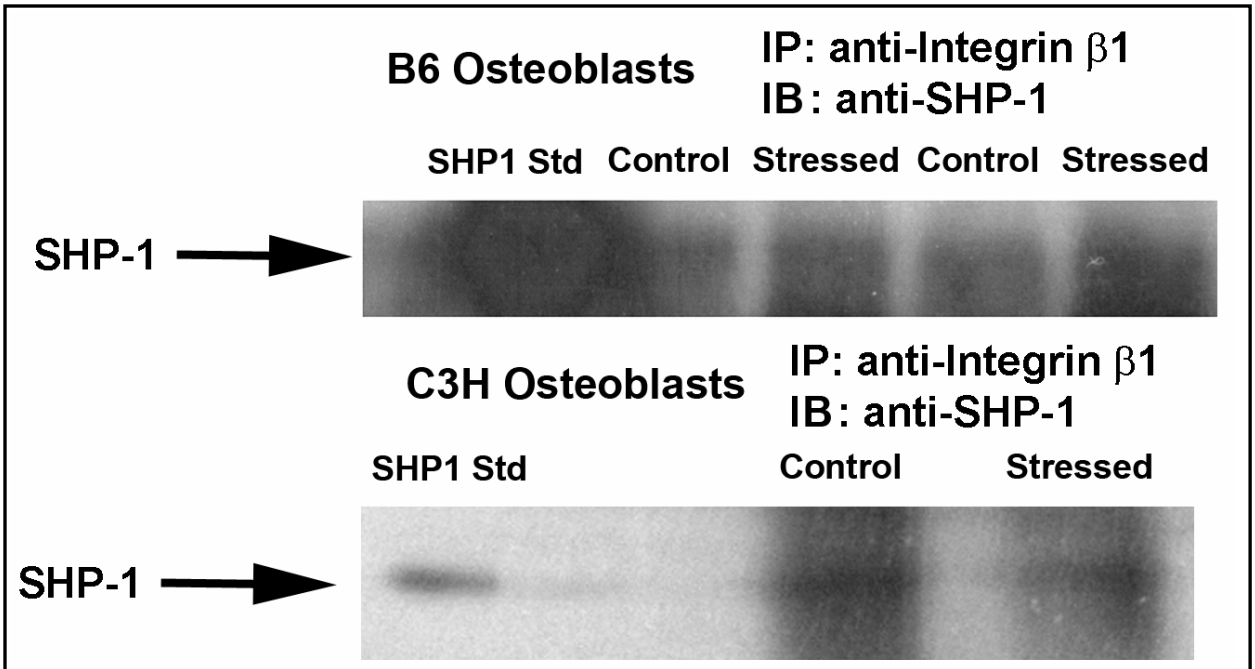

Figure 9. Fluid shear stress increased the amounts of integrin β1-associated SHP1 in B6 (top) but not in C3H (bottom) osteoblasts. Association of SHP1 with integrin β1 was shown by co-immunoprecipitation. Briefly, cellular proteins (~300 μg each) from static control and shear stressed B6 osteo-blasts and a single culture of C3H osteo-blasts were each immunoprecipitated (IP) with an antibody against mouse integrin β1 or a normal IgG. The precipit-ated proteins were resolved on a 10% SDS-PAGE, and the presence of SHP1 was identified by Western blots (IB) using an anti-SHP1 antibody. The 30-min shear stress increased the relative amounts of co-immunoprecipitated SHP1 by ~2-fold in B6 osteoblasts, but not in C3H osteo-blasts. No protein bands were seen in immunoprecipitates with non-immune IgG (not shown).

osteoblast extracts. The 30-min steady shear stress increased the amounts of co-immunoprecipitated SHP1 in B6 osteoblasts by ~2-fold, but appeared to reduce that in C3H osteoblasts. This preliminary experiment, albeit needed to be confirmed, supports the feasibility of the use of the co-immunoprecipitation approach to identify and quantify the integrin β1-associated cytosolic PTPs, such as SHP1, -2, and/or PTP1B. That the amounts of integrin β1-bound SHP1 in B6 osteoblasts was enhanced by the shear stress but appeared to be reduced by the same stress in C3H osteoblasts is consistent with the interpretations that fluid shear enhances the recruitment of cytosolic PTPs (e.g., SHP1) to integrin β1 and that the enhanced the integrin β1 recruitment of these PTPs in response to shear stress is impaired in C3H osteoblasts.

Key Research Accomplishments

1. We have obtained strong circumstantial evidence that the *Lepr* signaling pathway is a negative regulator of mechanical stimulation of bone formation.
2. We have shown that there are three SNPs in the coding region of *Lepr* between B6 and C3H mice, and that one of the SNPs resulted in the change of the amino acid sequence of the *Lepr* protein.
3. We have obtained preliminary evidence that leads to our hypothesis that the *Lepr* signaling pathway in osteoblasts negatively regulates the anabolic response to mechanical stimuli through sequestering SHP2 and/or PTP-1B from binding to integrins.

Reportable Outcomes

1. **Lau K-HW**, Kapur S, Amoui M, Wang X, Kesavan C, Mohan S, and Baylink DJ (2007) Leptin-deficient (ob/ob) mice exhibit increased bone mechanosensitivity and corresponding osteoblasts show increased anabolic shear stress responses in vitro. ***J Bone Miner Res*** 22 (Suppl 1), S23, abstract # 1081.

Conclusion

In summary, these data provide strong supports for our conclusions that 1) the *Lepr* and its signaling pathway acts as a negative regulator of mechanosensitivity, 2) the *Lepr* in osteoblasts of B6 and C3H mice might have different functional activity due to a SNP in the coding region, and the different functional activity of *Lepr* in osteoblasts of these two mouse strains may in part be responsible for the differential anabolic response to mechanical stimuli in these two mouse strains, and 3) the *Lepr* signaling pathway in osteoblasts negatively regulate the anabolic response to mechanical stimuli through sequestering SHP2 and/or PTP-1B from binding to integrins. We are now in position to apply the siRNA technology to definitively evaluate whether *Lepr* or its signaling mechanism is involved in the regulation of the osteogenic response to mechanical stimulation.

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Novel loci regulating bone anabolic response to loading: Expression QTL analysis in C57BL/6JXC3H/HeJ mice cross

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Abstract

Variations in the expression levels of bone marker genes among the inbred strains of mice in response to mechanical loading (ML) are largely determined by genetic factors. To explore this, we performed four-point bending on tibiae of 10-week female F2 mice of B6XC3H cross using 9N at 2 Hz, 36 cycles, once per day for 12 days. We collected tibiae from these mice for RNA extraction. We then measured the expression changes of bone marker genes, bone sialoprotein (BSP), alkaline phosphatase (ALP) and housekeeping genes, β-actin and peptidylprolyl isomerase A (PPIA), by using real-time PCR in both the loaded and the non-loaded tibiae of F2 mice (n=241). A genome-wide scan was performed using 111 micro satellite markers in DNA sample collected from these mice. Mean increase in gene expression, expressed as fold change, ranges from 2.8 to 3.0 for BSP and 2.7 to 2.8 for ALP. Both showed a skewed distribution with a heritability response of 87 to 91%. Absence of significant correlation between the increased gene expression vs. body weight (BW) and bone size (BS) suggests that bone response to loading is independent of BS or BW. Non-parametric mapping (MapQTL program 5) revealed that BSP and ALP expression in response to bending was regulated by several significant and suggestive QTL: Loci regulating both BSP and ALP were located on Chr 8 (60.1 cM), 16 (45.9 cM), 17 (14.2 cM), 18 (38.0 cM) and Chr 19 (3.3 cM); Loci specific to BSP were found on Chrs 1 (LOD score 10.4 at 91.8 cM), 5 (5.2 at 73.2 cM) and 9 (7.0 at 13.1 cM); Loci regulating only ALP were found on Chrs 1 (7.6 at 46 and 75.4 cM), 3 (8.3 at 47 cM) and 4 (5.6 at 54.6 cM). QTLs on Chrs 1, 3, 8, 9, 17 and 18 correspond to QTLs we previously reported by pQCT measurements, thus validating these findings. In addition, we found that the QTL associated with non-loaded tibiae for BSP and ALP on Chrs 4, 16 and 18 was identical to the QTLs associated with ML. This finding suggests that regions on these chromosomes are responsible for natural variation in expression of BSP and ALP as well as for ML. This is the first expression study to provide evidence for the presence of multiple genetic loci regulating bone anabolic response to loading in the B6XC3H intercross and will lead to a better understanding of how exercise improves the skeletal mass.

Keywords: Expression QTL; Mechanical loading; Inbred strains; Bone markers; Four-point bending

Introduction

Mechanical loading (ML) plays an important role in the maintenance of bone mass and strength. Several reports have provided evidence that mechanical loading stimulates bone formation and that immobilization or a loss of mechanical stimulation, such as bed rest or space flight, leads to a decrease in bone formation and an increase in bone loss [1,10,13,14,18,24,30,31,3,4,11,17,26,36]. Recent studies in humans have demonstrated that bone anabolic response varies widely among individuals when subjected to the same degree of mechanical load ranging from good to moderate response [7,9,27,29]. Analogously, experimental animals, particularly inbred strains of mice, have also shown variability with respect to mechanical loading. Studies have shown that there are greater fold changes in bone marker genes in C57BL/6J (B6) mice as compared with C3H/HeJ (C3H) mice when subjected to a same loading regimen [11]. It is likely that these variations in the bone anabolic response, in both human and mouse models, are due to

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differences in the transcription levels of genes, i.e., they are genetically controlled.

One of the approaches often used to study the genetic regulation of an observed phenotype is QTL mapping. This approach has been well-established in both human and mouse models and has revealed hundreds of chromosomal regions containing genes affecting bone phenotypes such as BMD, bone size and bone strength [2,15,22,23]. Previously, using this traditional or "classical" quantitative trait loci analysis (cQTL), we have identified several loci that regulate BMD and bone size in response to mechanical loading in the B6XC3H cross [12]. In order to validate these findings and to discover additional QTLs, we have used expression QTL mapping (eQTL) in the same inbred strain cross.

Recently, a number of studies in humans and animal models have provided evidence that expression levels of genes are amenable for genetic analysis in search of loci for the phenotypic variation [8,20,25,28,32,34,35]. This eQTL approach has several advantages: (1) it can map a QTL to the gene itself, indicating whether *cis* changes or *trans* factors are responsible for the different expression levels, (2) it allows one to identify genetic regions that directly control the expression levels of genes and (3) it validates that the chromosomal region identified from cQTL analysis and determines that these regions are responsible for the difference in transcription levels of genes whether responsible for the difference in bone response to loading between the two strains of mice. In the present study, we have treated expression levels of bone markers genes as quantitative traits for two inbred strains, C57BL/6J and C3H/HeJ, a good and poor responder, respectively, in order to perform a genome-wide search of loci regulating bone anabolic response to mechanical loading.

Materials and methods

Mice

Female B6 and male C3H mice were obtained from the Jackson Laboratory (Bar Harbor, ME) to produce C3HB6 F1 mice, which were intercrossed to generate F2 mice. All mice were housed under the standard conditions of 14-h light and 10-h darkness, and had open access to food and water. The experimental protocols were in compliance with animal welfare regulation and approved by local IACUC.

In-vivo loading model/regimen

Mechanical loading was performed using a four-point bending device (Instron, Canton, MA) on 10-week female F2 mice, following a previously reported protocol [11,12]. The mice were loaded using a 9 Newton (N) force at a frequency of 2 Hz for 36 cycles, once a day under inhaleable (5% halothane and 95% oxygen) anesthesia. The loading procedure was repeated for 6 days with 1 day of rest for 2 weeks. On the 15th day, mice were sacrificed and tibiae [loaded (bended bones) and non-external loaded (non-bended bones)] of the F2 mice were collected and stored in RNA later at −80 °C for further study.

RNA extraction

A Qiagen lipid extraction kit [Qiagen, Valencia, CA] was used to extract RNA from F2 bones with the following modification. After euthanization, tissues were removed from test mice, immediately transferred into liquid nitrogen and then stored at −80 °C until RNA extraction. Bones were ground into fine powder using mortar and pestle with liquid nitrogen. Approximately 1 ml of Trizol was added to each sample and ground until it became a fine powder. This fine bone powder was transferred to fresh 1.5 ml RNase free tubes. Chloroform (200 μl) was added to each sample, each sample was vortexed for 15 seconds (s) and incubated at room temperature for 3 min. The samples were then centrifuged at 12,000×*g* for 15 min and the aqueous layer was removed to a fresh tube after centrifugation. Approximately 700 μl of ethanol was added to the fresh samples and vortexed for 15 s. The samples were then transferred to a spin column and the RNA was purified according to the manufacturer's instructions. Quality and quantity of RNA were analyzed using Bio-analyzer and Nano-drop instrumentation (Agilent, CA, USA).

Reverse transcriptase–real-time PCR

Quantitation of messenger Ribonucleic Acid (mRNA) expression was carried out according to the manufacturer's instructions (ABIPRISM, Foster City, CA) using the SYBR Green method on 7900 Sequence Detection systems from Applied Biosystems. Briefly, purified total RNA (200 μg/μl) was used to synthesize the first strand cDNA by reverse transcription according to the manufacturer's instructions (Bio-Rad, CA). Five microliters of the five times diluted first strand cDNA reaction, was subjected to real-time PCR amplification using gene specific primers as described earlier [11]. The data were analyzed using SDS software, version 2.0, and the results were exported to Microsoft Excel for further analysis. Data normalization was accomplished using the endogenous control (β-actin, PPIA) to correct for variation in the RNA quality among samples. The normalized Ct values were subjected to a $2^{-\Delta\Delta}$Ct formula to calculate the fold change between the non-loaded and loaded groups. The formula and its derivations were obtained from the instrument user guide.

Genotyping

Genomic DNA extraction was extracted from the liver of each F2 mouse using a Maxi prep DNA extraction kit (Qiagen) and stored at −80 °C. Genotyping of these samples was performed as previously described [12].

QTL analysis

The broad sense of heritability index of each phenotype was calculated and a genome-wide analysis of the F2 population of B6XC3H cross was performed as previously described [12].

Statistical analysis

We used Statistica software (StatSoft, Inc version 7.1, 2005) to perform correlation analysis, phenotype distribution, regression analysis and two-way ANOVA. Significance levels were based on $p<0.05$.

Results

Expression levels of bone genes induced by loading are heritable

The bone anabolic response induced by mechanical loading in the F2 mice was measured by using two bone formation markers, bone sialoprotein (BSP) and alkaline phosphatase (ALP). Expression levels from each of the markers were measured as fold change by comparing the difference between loaded tibiae vs. non-loaded tibiae. The mean fold increases in BSP and ALP, in the parents, female F1 and F2 mice, normalized by β-actin and by PPIA are shown in Table 1. The fold change data for the BSP and ALP marker genes in the female F2 mice, obtained after normalizing with β-actin and PPIA, show skewed distribution (Fig. 1). The skewed distribution appears to be due to the fold change calculation which amplifies the difference geometrically

Table 1
Fold changes in the expression levels of bone marker genes in response to 12 days four-point bending on 10-week female mice

Groups	BSP	ALP	*N*
B6 parents	8.41±0.76	6.29±0.71	5
C3H parents	2.93±0.62	3.38±0.69	5
F1 β-actin normalized	1.87±2.36	2.09±2.03	16
PPIA normalized	1.44±1.82	1.82±1.89	
F2 β-actin normalized	3.0±2.59	2.82±2.52	241
PPIA normalized	2.83±2.31	2.70±2.31	

Values mentioned above are mean±SD of the fold change.

($2^{-\Delta\Delta CT}$). Thus, by adjusting the data with natural log, the distribution became normal. The broad sense of heritability, calculated as described previously, for the loading-induced fold changes in the BSP and ALP, after normalizing with β-actin was 87% and 91%, respectively, and after normalizing with PPIA was 88% and 91%, respectively, in the F2 population.

We next determined whether the mRNA levels of bone formation marker genes (fold change), measured by real-time PCR, correlate with the changes in BMD and log PC measured by pQCT. The results show a significant positive correlation between fold changes and BMD (r=0.25 to 0.30), and bone size (r=0.27 to 0.36). These findings suggest that these two bone formation markers are responsible, in part, for the increase in BMD and PC in response to loading in the F2 mice.

Since B6 and C3H mice exhibit difference in bone size due to their genetic background, we expect a variation in the cross-sectional area among F2 mice. Due to this variation, mice with smaller cross-sectional areas are predicted to receive a higher mechanical strain, while mice with larger cross-sectional areas to receive less mechanical strain to the same load. In order to determine if this variation in strain affected the expression levels of these markers, we performed a correlation analysis between the fold changes in BSP and ALP, normalized with β-actin and PPIA, and non-loaded PC measurements of each F2 mouse. These results showed no correlation (r=−0.003 to 0.03). Similarly, we found no correlation between body-weight and fold change data for BSP and ALP. These data suggest that variation in the increase in mRNA levels of BSP and ALP genes induced by mechanical loading is largely independent of bone size and body weight in the B6XC3H intercross.

Expression QTL for bone formation response induced by loading

Using 111 micro-satellite markers and loading-induced fold change data of BSP and ALP marker genes of F2 female mice (n=241), a genome-wide analysis revealed the presence of significant and suggestive genetic loci affecting bone anabolic response (Table 2). Loci regulating both the expression of BSP and ALP, normalized with β-actin and PPIA, were located on

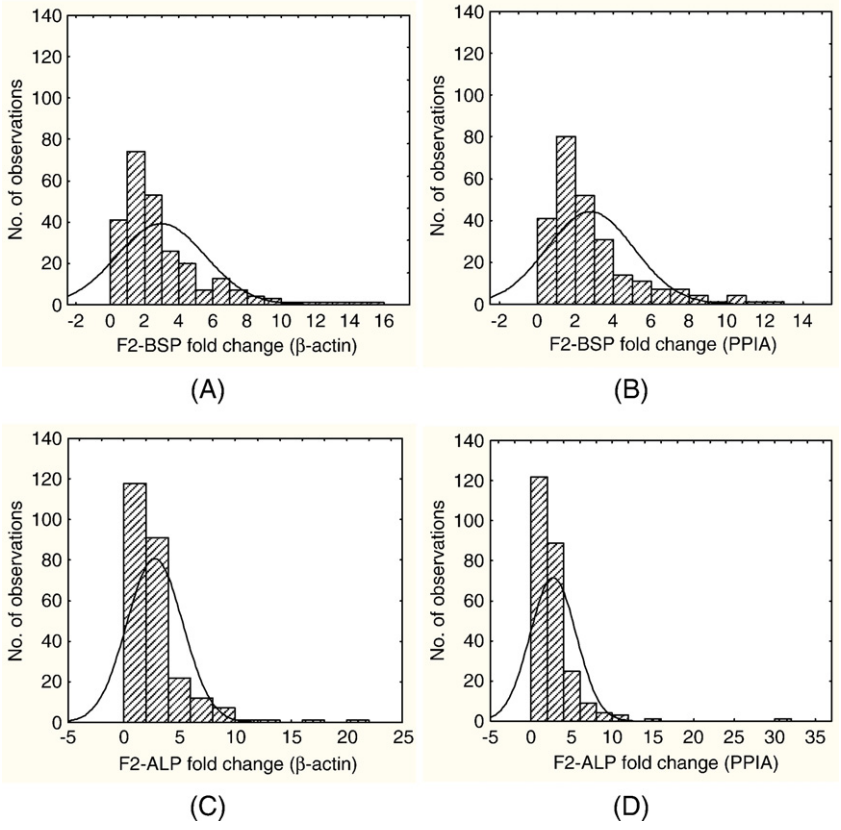

Fig. 1. Distribution of fold changes for (A) BSP normalized with β-actin, (B) BSP normalized with PPIA, (C) ALP normalized with β-actin and (D) ALP normalized with PPIA in the F2 population after 2 weeks of four-point bending. The *x*-axis represents the fold change and *y*-axis represents the number of observations (mice). BSP, Bone sialoprotein; ALP, Alkaline phosphatase; β-actin, Beta actin and PPIA, Peptidy-prolyl *cis–trans* isomerase A. The solid line represents theoretical skewed distribution. Based on Kolmogorov–Smirnov test, both BSP and ALP fold change data show skewed distribution (n=241).

Table 2
Significant and suggestive QTL identified using fold change data for the mechanical loading-induced phenotypes in the B6XC3H F2 mice

Phenotypes	Chr	Locus	cM	Actin normalization	PPIA normalization
				LOD score	LOD score
Bone sialoprotein	1	D1Mit113	91.8	–	10.4[a]
	5	D5Mit143	73.2	–	5.2[c]
	8	D8Mit88	60.1	7.5[b]	8.8[b]
	9	D9Mit2	13.1	7.0[b]	4.8[c]
	9	D9Mit151	69.9	7.1[b]	5.0[c]
	16	D16Mit153	45.9	6.7[b]	7.0[b]
	17	D17Mit51	14.2	9.2[a]	12.3[a]
	18	D18Mit144	38	8.0[b]	–
	19	D19Mit68	3.3	7.2[b]	10.7[a]
Alkaline phosphatase	1	D1Mit215	47	5.0[c]	7.5[b]
		D1Mit102	75.4	6.3[b]	7.6[b]
	3	D2Mit147	59	7.7[b]	8.3[b]
	4	D4Mit308	54.6	5.5[c]	5.6[b]
		D4Mit256	82	5.6[c]	5.0[c]
	8	D8Mit88	60.1	10.5[a]	12.3[a]
	9	D9Mit151	69.9	7.4[b]	–
	16	D16Mit153	45.9	5.1[c]	5.9[c]
	17	D17Mit51	14.2	9.3[a]	9.0[b]
	18	D18Mit144	38	12.6[a]	5.8[c]
	19	D19Mit68	3.3	–	6.9[b]

[a]The threshold for the highly significant LOD score is $p<0.01$.
[b]The threshold for the significant LOD score is $p<0.05$.
[c]The threshold for the suggestive LOD score is $p<0.1$.
–Corresponds to no QTL.

Chromosomes 8, 9, 16, 17, 18 and 19. Loci regulating only BSP were located on Chrs 1, 5 and 9, whereas loci regulating only ALP were located on Chrs 1, 3 and 4. For BSP, highly significant LOD scores were observed on Chr 1 (LOD score 10.4 at 91.8), Chr 17 (LOD score 12.3 at 14.2 cM) and Chr 19 (LOD score 10.7 at 3.3 cM). For ALP, highly significant LOD scores were observed on Chr 8 (LOD score 12.3, at 60 cM), Chr 17 (LOD score 9.3, at 14.2) and Chr 18 (LOD score 12.6 at 38 cM).

Expression QTL for housekeeping genes

To assure that the QTLs identified for BSP and ALP fold change in response to mechanical loading are not due to changes in the housekeeping genes, we calculated fold change of β-actin normalized by PPIA and PPIA fold change normalized by β-actin. We found that the mean fold difference in β-actin was 0.99±0.30 and in PPIA was 1.05±0.33 in the F2 mice. Interval mapping using F2 mice ($n=241$) revealed three suggestive QTL on chromosomes 1, 9 and X and one significant QTL on Chr 2 (Table 3). We found that one of the loci on Chr 9 (69.9 cM) identified for the β-actin fold change data corresponds to one of the loci identified for both BSP and ALP fold change data. This finding leads us to suspect that the QTL identified for ALP and BSP on Chr 9 could be due to expression changes in the house keeping gene rather than due solely to expression changes in the marker genes.

Table 3
Interval mapping for the fold change in β-actin (normalized by PPIA) and PPIA (normalized by β-actin) in response to mechanical loading in the B6XC3H F2 mice

Phenotypes	Chr	Locus	cM	LOD score	Variance
β-actin	1	D1Mit430	6.6	2.0[c]	3.6
	2	D2Mit66	48.1	2.8[b]	5.4
	9	D9Mit151	69.9	1.9	3.6
PPIA	2	D2Mit285	72.1	*	3.3
	X	DXMIt172	40.4	2.1[c]	4.7

[b]The threshold for the significant LOD score is $p<0.05$.
[c]The threshold for the suggestive LOD score is $p<0.1$.
*QTL with very low LOD score.
Variance is explained from peak LOD score.

QTL for BSP and ALP in non-external loaded tibia

In addition to the mechanical loading QTL, we identified QTLs that regulate the basal expression of BSP and ALP in the non-loaded tibiae, by using Ct values from real-time PCR, after normalization with β-actin and PPIA. Both BSP and ALP showed normal distribution in the F2 population (Fig. 2). We found that the BSP and ALP data showed no correlation with body weight suggesting that the basal expression of these two bone formation marker genes is independent of the body weight. Interval mapping was then performed using F2 female mice ($n=241$), which revealed four chromosomes, Chrs 4, 10, 16, and 18, that regulate the basal expression of BSP and ALP in the non-loaded tibiae (Table 4).

Discussion

The salient features of the present study are: (1) Fold change measurements in bone marker expression led to the identification of several QTLs which regulate bone adaptive response to

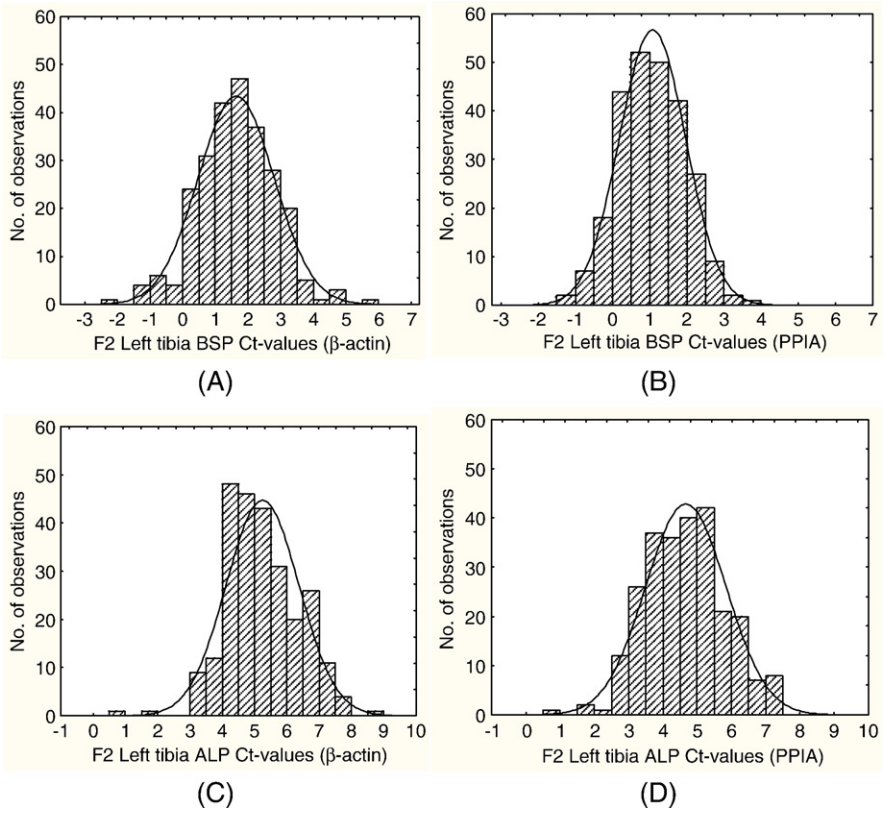

Fig. 2. Distribution of Ct values of non-external loaded tibia for (A) BSP normalized with β-actin, (B) BSP normalized with PPIA, (C) ALP normalized with β-actin and (D) ALP normalized with PPIA in the F2 population after 2 weeks of four-point bending. The *x*-axis represents the fold change and *y*-axis represents the number of observations (mice). BSP, Bone sialoprotein; ALP, Alkaline phosphatase; β-actin, Beta actin and PPIA, Peptidy-prolyl *cis–trans* isomerase A. The solid line represents theoretical normal distribution. Based on Kolmogorov–Smirnov test, both BSP and ALP show normal distribution ($n=241$).

loading. (2) We found QTLs common to both ALP and BSP as well as QTLs specific to ALP but not BSP or vice versa. (3) We found that some of the expression QTLs matched the classical QTL we previously identified for BMD and bone size validating our findings.

We undertook a number of precautions to ensure that the QTLs we identified are real and not due to technical or design artifacts: (1) We chose two markers, BSP and ALP, that showed significant positive correlation in bone anabolic response to loading in a previous study [11]. (2) We used fold changes rather than Ct values to study linkages so that we would identify specific genetic changes rather than general changes. (3) We used two housekeeping genes, rather than one, to normalize the expression data in order to avoid identification of QTLs stemming from variations in RNA quality among samples. For example, loci on Chrs 9 and 18 show significant linkage with ALP and BSP, respectively, using β-actin, but not PPIA as an internal control. Thus, the validity of Chr 9 and 18 QTL remains to be established.

Our linkage analysis revealed several QTLs that are responsible for the increased expression levels of BSP and ALP,

Table 4
Significant and suggestive QTL identified using Ct values for the non-externally loaded phenotypes in the B6XC3H F2 female mice

Phenotypes	Chr	Locus	cM	LOD score	Variance	LOD score	Variance
				Actin normalization		PPIA normalization	
Bone sialoprotein	4	D4Mit42	76.5	2.7[b]	5.2	2.4[c]	4.5
	18	D18Mit64	0	2.4[c]	4.6	*	2.3
Alkaline phosphatase	4	D4Mit42	76.5	2.8[b]	5.2	*	3.3
	10	D10Mit213	6.6	2.3[c]	4.3		
	16	D16Mit153	45.9	1.9	3.6	2.3[c]	4.2
	18	D18Mit144	38.3	2.4[c]	6.7	*	2.3

[b] The threshold for the significant LOD score is $p<0.05$.
[c] The threshold for the suggestive LOD score is $p<0.1$.
* QTL with very low LOD score.
Variance is explained from peak LOD score.

induced by mechanical loading in the F2 mice. If changes in BSP and ALP markers reflect skeletal changes to mechanical loading, one would expect QTLs which are common to both markers. Accordingly, we found co-localized loci on Chrs 8, 16, 17, 18 and 19 for both BSP and ALP, suggesting that both markers are responding to the same upstream signaling.

Our findings also revealed four loci on Chrs 3, 8, 17 and 18 which are identical to QTLs we previously found for BMD and/or bone size parameters [12]. This is consistent with other studies which have shown that Chrs 8 (30–90 cM), 17 (6.6 cM) and 18 (32–46 cM) contain a loci which regulate biomechanical properties in several inbred mouse strain crosses [15,16,21,22]. The fact that we found QTLs at the same loci using both bone parameters and bone formation markers, indicate that these loci do, in fact, contain genes that are not only involved in increasing bone formation in response to loading, but also involved in regulating mechanical properties of the bone, in part, through ALP and BSP expression. While the expression QTLs found on Chr 17 and 18 were contained within the region identified for the BMD and bone size, the broad QTL regions in these chromosomes raise the possibility that more than one gene could be responsible for the phenotypic changes. Fine mapping will narrow down the size of the QTL and allow us to identify it as the same QTL as identified for BMD or as a different QTL. Surprisingly, we identified additional QTLs for BSP and/or ALP which do not correspond with any QTLs reported for bone parameters. This could be explained by: (1) changes in gene expression might be more sensitive to external loading than net change in the bone parameters (measured by pQCT) and (2) these regions may be involved in regulating the expression of BSP or ALP, but have no measured effect on bone formation.

The QTLs identified in this study common to both BSP and ALP are more than 10 cM in length, and thus, contain hundreds of genes and ESTs. Some of the known candidate genes located in these QTL regions are shown in Table 5. We have previously reported that the expression of some of these genes increases with mechanical loading using a genome-wide microarray analysis [33]. Others using various approaches have shown that many of these genes are involved in skeletal development. This confirms our present QTL findings. Although QTL analysis leads to a precise mapping of the genetic loci which contribute to our phenotype of interest, these regions are broad and contain many possible significant genes. The next phase of our study, therefore, lies in identifying which specific genes within our identified QTL regions are associated with mechanical loading.

Some of the limitations of this study are: (1) we used a relatively small number of F2 mice to perform the eQTL analysis ($n=241$) relative to the cQTL analysis ($n=329$). This is due to fact

Table 5
List of potential candidate genes located in the QTL region identified for skeletal anabolic response to mechanical loading

Chrs	cM	Genes	Predicted functions in bone
8	38–69	Ptger1	Prostaglandin E2 stimulates fibronectin through ptger1
		Junb	Involved in osteoblast cell proliferation
		Mt1, 2	Regulate early stage of mesenchymal stem cells differentiation
		Cdh11	Knock out (KO) mice show reduced bone density
		Hsd11b2	Regulate glucocorticoid signaling
		Cdh1	Important in embryonic limb buds development
		Cbfb	Required for the function of Runx1 and Runx2 in skeletal development
		Hsd17b2	Involved in regulation of estrogen actin
		Il17c	Involved in osteoclastogenesis
16	30–46	Col8a1	Increased in bone in response to mechanical loading
		EphA3	Involved in tooth development
		Pit1	Mediates bone formation by regulating the expression of BSP
17	0–25	Map3k4	Involved in normal skeletal patterning
		Clcn7	Critical for osteoclast resorption
		Thbs2	KO mice show increased bone density and cortical thickness
		Traf7	Involved in MEKK3 signaling and apoptosis
		Tnf	Important in osteoclastogenesis
		Notch3	Involved in tooth development
		Vegfa	Involved in angiogenesis of bone
		Runx2	Involved in skeletal development
18	15–40	Lox	Play a key role in the collagen deposition by osteoblast
		Pdgfrb	Involved in bone remodeling phase
		Adrb2	Produces anabolic effects on bone
		Mc4r	KO mice show decreased bone resorption and high bone mass
		Mapk4	Intracellular mediator of growth factor
		Nfatc1	Important in osteoclastogenesis
		Galr1	Important for bone healing
19	0–24	Gal	Involved in bone healing
		Lrp5	Regulates osteoblast function
		Esrra	Important in bone metabolism
		Lpxn	Involved in podosomal signaling complex in osteoclast
		Ostf1	Stimulates osteoclast formation
		Jak2	Involved in osteoblast signaling

that we encountered problems with quality and quantity in the RNA extracted from 6 mm, marrow-flushed tibiae. This relatively small sample size may account for the reduced LOD score for some of the QTLs identified in our study. (2) The QTLs identified in this study for bone formation response induced by mechanical loading were obtained from female mice. To date, studies have shown that hormones enhance the effects of mechanically induced bone formation [5,6,19]. Further studies with male mice may not only reveal whether any of the QTLs we found are female specific but may also lead to the identification of male specific QTLs involved in bone response to mechanical loading. (4) It has been predicted that some of the skeletal changes in the F2 mice could be due to periosteal pressure caused by four-point bending. Our previous findings that sham loading neither increased periosteal bone formation nor caused changes in expression levels of bone formation marker genes (data not shown) argue against this possibility [12].

Conclusion

We provide evidence that bone formation response varies among individuals and is strongly regulated by genetic factors, as is evident from both our previous cQTL and our present eQTL analysis. From both the QTL analyses, we show that Chr 8 contains genes involved in increasing bone formation induced by mechanical loading and for biomechanical properties of the bone. Further study, with congenic and gene KO mice may help to understand the role of specific genes at the loci we have found and could provide a basis for understanding the observed variability in bone mass accretion and maintenance, resulting from exercise, in normal healthy individuals.

Acknowledgments

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Identifying Mechanical Loading QTL by Gene Expression Changes for Alkaline Phosphatase and Bone Sialoprotein in C57BL/6J (B6) X C3H/HeJ (C3H) Intercross. C. Kesavan, D. Baylink*, S. Kapoor*, S. Mohan, MDC, J.L. Pettis VAMC, Loma Linda, CA, USA.

Previous studies have shown that mechanical loading (ML) 1) produces a more robust skeletal anabolic response in B6 mice than C3H mice, which is largely mediated by genetic differences and 2) induces greater expression of bone formation (BF) marker genes in the bones of B6 mice compared with C3H mice. We, therefore, tested the hypothesis that the variation in skeletal anabolic response to ML in the F2 mice of B6 and C3H intercross is largely due to changes in BF response. To examine this, we measured expression changes in two BF markers, namely bone sialoprotein (BSP) and alkaline phosphatase (ALP) in the loaded and non-loaded bones of 10-week F2 female mice (n=241). Loading was performed daily on the right tibia by four-point bending using a 9 Newton at 2Hz, 36 cycles, for 12 days. The left tibiae were used as internal controls. The expression levels of ALP and BSP were quantitated after normalization with two house keeping genes, actin and PPIA. The mean increase in gene expression in the F2 mice, expressed as fold change, ranged from 3.0 ± 2.8 for BSP and 2.7 ± 2.8 for ALP, and showed significant correlation (r=0.25 to 0.36, p<0.01) with changes in BMD and bone size. A genome-wide search using 111 microsatellite markers with 15 cM intervals in the

Locus *	β-actin Normalization		PPIA Normalization	
	BSP-LOD	ALP-LOD	BSP-LOD	ALP-LOD
D8Mit88	7.5[b]	10.5[a]	8.8[b]	12.3[a]
D9Mit151	7.1[b]	7.4[b]	5.0[c]	-
D16Mit153	6.7[b]	5.1[c]	7.0[b]	5.9[c]
D17Mit51	9.2[a]	9.3[a]	12.3[a]	9.0[b]
D18Mit144	8.0[b]	12.6[a]	-	5.8[c]
D19Mit68	7.2[b]	-	10.7[a]	6.9[b]

* determined using MapQTL program; [a]p<0.01, [b]p<0.05, [c]p<0.1

F2 mice revealed QTLs on Chrs 8, 9, 17 and 18, which corresponded to ML QTLs we previously identified using changes in BMD and bone size as end points. We identified two new ML QTLs on Chrs 16 and 19 using gene expression data. In conclusion: 1) Identification of several common QTL for BMD, BSP and ALP phenotypes suggests that the skeletal response to ML is largely mediated by increased BF; 2) Identification of genes that are involved in regulating BSP and ALP expression in response to ML will lead to improved understanding of the molecular pathways regulating the bone response to ML.

Disclosures: C. Kesavan, None.

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Leptin-deficient (ob/ob) Mice Exhibit Increased Bone Mechanosensitivity and Corresponding Osteoblasts Show Increased Anabolic Shear Stress Responses In Vitro. K. H. W. Lau[1], S. Kapur*[1], M. Amoui*[1], X. Wang*[1], C. Kesavan*[1], S. Mohan[1], D. J. Baylink[2]. [1]Loma Linda VAMC, Loma Linda, CA, USA, [2]Loma Linda Univ., Loma Linda, CA, USA.

We sought to test the hypothesis that the leptin receptor (Lepr) pathway plays an important role in bone mechanosensitivity based on the rationales that Lepr is located within one of the mouse genetic loci that showed mechanosensitivity modulating effects and that Lepr signaling is essential for skeletal maturation and metabolism. To test this hypothesis, the osteogenic response to loading (in the form of 2-week four-point bending) in tibia of adult female ob/ob mice [in C57BL/6J (B6) background] was compared with those in adult female B6 tibia. To adjust for the 14% greater bone size in ob/ob mice, the load was adjusted to produce similar levels of mechanical strain (2129 με at 9N for ob/ob mice vs. 2500 με at 6N for B6 mice). This mechanical strain was insufficient to produce a bone anabolic response in B6 mice; whereas in ob/ob mice this strain increased total BMC (16%), cortical area (22%), content (29%), and thickness (28%), and BMD (8%) [$p<0.05$ for each]. To further test if leptin deficiency would enhance osteogenic response to mechanical stimuli, the effects of a 30-min fluid shear (20 dynes/cm^2) on [^{3}H]thymidine incorporation (TdR) and Erk1/2 phosphorylation in ob/ob osteoblasts were compared to those of wild-type (WT) littermates and B6 mice. While the shear stress increased TdR and Erk1/2 in osteoblasts of B6 and WT littermates (each by ~2-fold, $p<0.05$), the stimulation in ob/ob osteoblasts was greater (> 3-fold, $p<0.05$ vs. B6 osteoblasts). In addition, 2-hr pretreatment of ob/ob osteoblasts with 100 ng/ml of leptin completely abrogated the enhanced mitogenic response. Because it has been reported that the mechanism whereby mechanical stimuli act to stimulate proliferation involves upregulation of genes of the IGF-I, Wnt, BMP/TGFβ, and estrogen receptor pathways, we next determined whether the Lepr pathway acts upstream to these 4 pathways by assessing the effects of the fluid shear on the expression levels of representative genes of these pathways in ob/ob and B6 osteoblasts (by real-time PCR). The upregulation of each test gene of the 4 pathways in ob/ob osteoblasts was much greater ($p<0.05$) than those in B6 osteoblasts. The 2-hr leptin pretreatment also abrogated the shear stress-induced upregulation of these genes in ob/ob osteoblasts. Conclusions: 1) In vivo, the Lepr pathway inhibits the anabolic responses to mechanical loading which is consistent with increased basal values for bone size in the ob/ob mouse, 2) In vitro, the Lepr pathway also has a negative modulating role in the mechanosensitivity of mouse osteoblasts, and 3) The Lepr pathway acts upstream of 4 selected major anabolic pathways to modulate mechanosensitivity.

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